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THE JOURNAL OF GENERAL MICROBIOLOGY

EDITED FOR
THE SOCIETY FOR GENERAL MICROBIOLOGY

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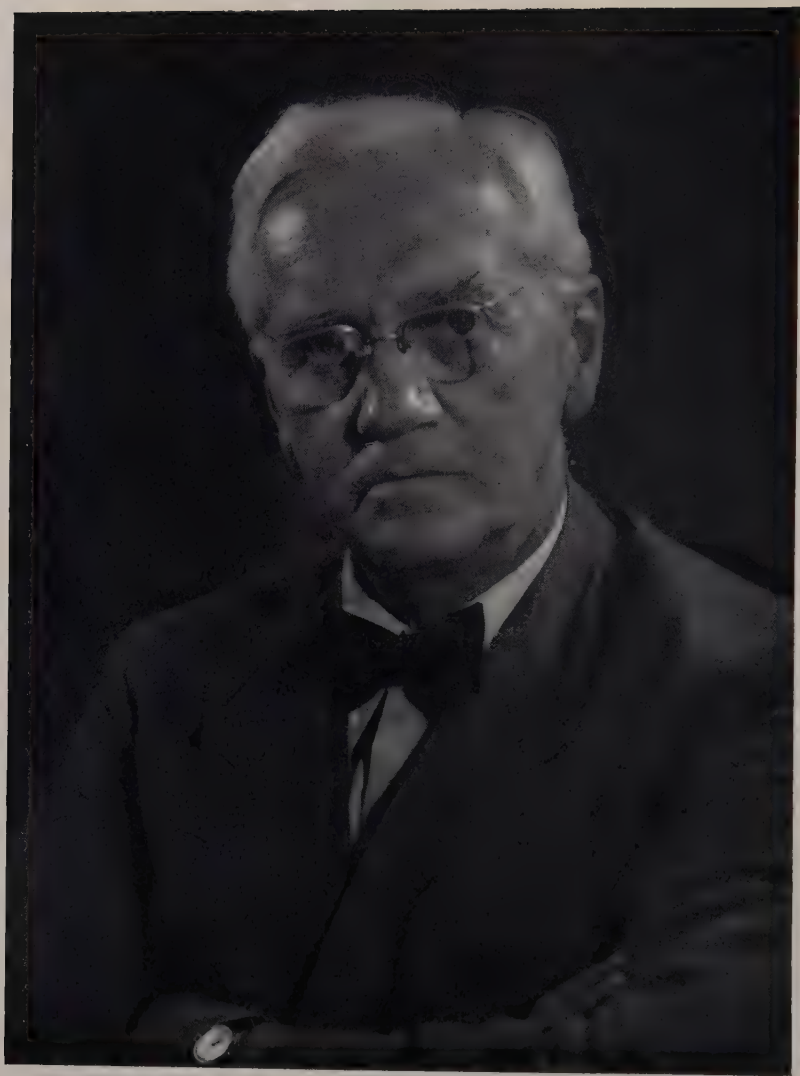
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SIR ALEXANDER FLEMING (1881-1955)

Obituary Notice

SIR ALEXANDER FLEMING, 1881-1955

By the sudden death of Sir Alexander Fleming at his home in London, on 11 March 1955, the world has lost one of the most outstanding and best-known figures of the present century. It is given to few during their lifetime to have a world-wide reputation and to command universal respect and admiration, but it was undoubtedly given to Fleming for his discovery of penicillin. The honours and rewards that began to be showered on him from all over the world, more than sixteen years after his original discovery, he accepted with the innate quiet modesty that was typical of the man.

The youngest of the eight children of an Ayrshire farmer, Fleming was born at Lochfield Farm, near Darvel, on 6 August 1881. His father died when he was only 7 years of age. Fleming received his first schooling at Loudoun Moor School; at the age of 10 he went to the village school at Darvel, and two years later he continued his education at Kilmarnock Academy. When he was 14 he joined an elder brother in London where he continued his education for two years at the Polytechnic, Regent Street; then in 1897 he took a junior post in the offices of a shipping company in the City, where he worked for four years. In 1901 his share of a small legacy enabled him to reconsider his future, and with encouragement from his brother he returned to his studies, passed the London Matriculation and decided on a medical career. He chose to enter St Mary's Hospital Medical School, simply because he had played water-polo against St Mary's. He easily won the Senior Entrance Scholarship in Natural Science and entered St Mary's in 1902. During his undergraduate career he won almost all the scholarships and class prizes: he obtained his medical qualification under the Conjoint Board in 1906, and in 1908 he graduated M.B., London University, with honours in five subjects, and won a University Gold Medal. A year later he took the F.R.C.S. His memory was described by his contemporaries as phenomenal, and learning came easily to him. In 1900 he had joined the London Scottish Volunteers and represented the London Scottish Shooting Team when they won the *Daily Telegraph* Cup at Bisley in 1908, two days before his M.B. examination. As a student he was a regular member of the hospital shooting team, the water-polo team, and took part in amateur theatricals.

In 1902, Sir Almroth Wright had been appointed to the Chair of Pathology at St Mary's Hospital Medical School, and Fleming first came into contact with him in 1906 as pupil and assistant in the Inoculation Department. Immunology was at this time in the forefront in bacteriological laboratories, and the controversy between two opposing schools of thought—the 'cellular theory' of immunity with Metchnikoff and his disciples as the main protagonists, and the 'humoral theory' represented by Koch, von Behring, Pfeiffer

and others—was at its height. Wright and Douglas had already published several papers on the phagocytic power and opsonic power of the blood in skin infections with staphylococci, and Fleming, influenced by Wright's personality, energy and fertility of ideas, and by the attraction of research work, entered Wright's laboratory immediately after he qualified in 1906. Here he worked in close association with Wright and his team of workers till the death of Wright in 1947, when Fleming succeeded him as Director of the Inoculation Department, which latterly became the Wright-Fleming Institute.

Wright and Douglas, ably assisted by other members of the team, including Colebrook and Parry Morgan, were developing new techniques for the measurement of the bactericidal power of the blood and of the number of viable organisms in cultures. In the development, elaboration and use of these new techniques, based on the microscope slide and Pasteur pipette, Fleming played a full part, and his invaluable help was recognized with the publication by Wright and Colebrook in 1912 of *The Technique of the Teat and Capillary Glass Tube*.

In April 1908, Fleming and another member of Wright's team, Dr Leonard Noon, published an article in the *Lancet* on 'The Accuracy of Opsonic Estimations', and the first paper under his name alone appeared in *The Practitioner* in May of the same year—this dealt with the accuracy of methods used for estimation of the opsonic index and sources of error to be avoided.

During the next few years Fleming developed a modified method for carrying out the Wassermann test, which eliminated the use of haemolytic amboceptor prepared by immunizing rabbits with sheep red cells, and substituted the haemolysin for sheep red cells normally found in fresh human serum (1909*a*). This technique was widely used for many years and was later combined with the Harrison technique. In the same year (1909*b*) he described a simple medium for the growth and isolation of the acne bacillus, consisting of ordinary nutrient agar with the addition of 1–5% of oleic acid. Subsequently, the successful treatment of cases of acne with autogenous vaccines was reported. Fleming & Colebrook were the first (1911) in Great Britain to test and report on the use of Ehrlich's newly discovered Salvarsan (606) for the treatment of syphilis, following the gift of a supply for trial from Ehrlich to Wright; the results were described as remarkable and no toxic or other effects were noted following intravenous use. This was the beginning of chemotherapy, and the beginning of Fleming's interest in the investigation of chemical antiseptics in the treatment of infection, an interest which became the mainspring of his future work.

During the first World War, Fleming served in the R.A.M.C. and worked with Wright and his team in a laboratory set up in Boulogne. Here they investigated the infections of war wounds and new methods of treatment, and many of the techniques evolved for the study of the physiology and immunology of infected wounds were devised by Fleming. The lack of value, and even the harmful and sometimes toxic effects of antiseptic packs for treatment of war wounds was soon realized, and the destructive effect of phenol and other antiseptics on the bactericidal power of the blood was

clearly demonstrated (1919) by Fleming in his classical experiment with *Bacillus perfringens* (*Clostridium welchii*). Similarly, he showed that a solution of sodium hypochlorite (Dakin's fluid) used to dress or irrigate war wounds was only transiently of value as an antiseptic, and that its main good effect was to increase the amount of exudate from the walls of the wound, an effect which could equally well be produced by the application of hypertonic saline solutions. Later he showed that flavine and acriflavine, widely used dyestuff antiseptics, had a highly destructive effect on leucocytes in the strengths recommended for use in wounds, that they delayed wound repair, and failed to sterilize infected wounds. In further experiments made some years later (1924, 1940b), using the slide-cell technique, he noted that the antileucocytic effect of acriflavine in blood was not so great as his original observations had led him to believe, and that, for short periods at least, leucocytes could retain motility and phagocytic power in the presence of concentrations of acriflavine regarded as being antiseptically effective in the treatment of infected wounds. Another ingenious experiment devised by Fleming, the so-called 'artificial wound', illustrated the difficulty of ridding an infected wound of its organisms by means of antiseptics; this consisted of a test-tube with several conical spikes drawn out in its lower half, filled with serum and infected with faecal matter; the serum heavily infected after incubation was replaced by antiseptic solution which was in turn after some hours replaced by fresh sterile serum. This procedure, repeated for several days, failed to sterilize the contents, due to failure of the antiseptic to penetrate to the tips of the glass spikes, called by Wright 'ecphyllactic foci' and corresponding to crevices and pockets in a wound, inaccessible to antiseptics. During the course of investigations of gas-gangrene due to *C. welchii* in war wounds, Fleming found that the organism, although an obligate anaerobe, would grow under aerobic conditions if a piece of asbestos, fabric or potato were added to the fluid medium—an *in vitro* reproduction of the conditions pertaining in many wounds in which fragments of clothing had become embedded. With Douglas & Colebrook (1917) he also showed the important role played by bacterial symbiosis in wound infections, especially in the occurrence of gas-gangrene. It is of interest that in 1915 Fleming, in a paper on the bacteriology of septic wounds, recommended *débridement* for the treatment of severe wounds as a means of reducing infection, a method which became a standard surgical procedure in the second World War, over 30 years later. He must also have been one of the first to suggest, in 1919, in a joint paper with Porteous, that infection of war wounds with *Streptococcus pyogenes* was in the great majority of cases due to cross-infection in hospital, a deduction that was amply to be confirmed some 15 years later, when serological typing of *S. pyogenes* enabled the sources and paths of spread of infection with this organism to be traced with considerable precision.

After the war in 1918, Fleming returned to St Mary's as assistant to Sir Almroth Wright and was also appointed Lecturer in Bacteriology in St Mary's Hospital Medical School. In 1920 he became director of the Department of Systematic Bacteriology and assistant director of the Inoculation Department.

For some years he also acted as pathologist to the London Lock Hospital and to the venereal diseases department at St Mary's. He continued his investigations on the action of antiseptics in septic wounds, and in a series of experiments produced evidence in support of the 'physiological' school of Wright, the aim of which was to aid the natural protective agencies of the body against infection, and showed that the treatment of septic wounds with chemical agents, practised by the 'antiseptic' school, was not only ineffective in sterilizing wounds but inhibited or killed the leucocytes, and under certain conditions stimulated the growth of the infecting organisms. The evidence showed that all antiseptics in use at that time damaged the body cells, when used at a concentration necessary to kill the infecting organisms.

Towards the end of 1921, shortly after the writer began to work with Fleming, the latter made a discovery to which he later ascribed more importance than his discovery of penicillin. This was lysozyme, 'a substance present in the tissues and secretions of the body, which is rapidly capable of dissolving certain bacteria'. The circumstances leading to this discovery were in many respects similar to those which led to his discovery of penicillin, seven years later. His investigations of the nature and properties of lysozyme formed the subject of his first contribution to the Royal Society in February 1922. Subsequent investigations (1922*a, b*) convinced him of the enzyme-like nature of lysozyme and its importance as a factor contributing to natural immunity, acting as one of the body's natural defences against infection. Two techniques in particular which he adapted to his investigations on lysozyme were the slide-cell devised by Wright for investigating the bactericidal power of whole blood, and the agar plate with a gutter or a cup punched out with a cork-borer, later used with modifications in the assay of penicillin and other antibiotics.

Coincidental with his investigations on lysozyme, Fleming continued his experiments on the activities of antiseptics on bacteria and leucocytes, experiments in which the slide-cell played a large part, and he showed that the antiseptics then in common use, iodine, phenol, picric acid, mercuric chloride, eusol, alcohol, ether, and flavine, could never be successfully introduced into the blood stream for the treatment of septicaemia, nor indeed were they effective in the treatment of septic wounds—time and experience have fully confirmed his views.

In 1928 Fleming was appointed Professor of Bacteriology in the University of London, the post being tenable at St Mary's, and in September of the same year he made the discovery of penicillin, which was not to see full fruition till 1941, during the second World War. The circumstances of the discovery have been repeated so often that it suffices to say that it occurred as an observation made while he was working with colony variants of staphylococci with a view to writing an article for the Medical Research Council's 'System of Bacteriology' (1929*a*)—'it was noticed that around a large colony of contaminating mould the staphylococcus colonies became transparent and were obviously undergoing lysis'. This simple observation, and the subsequent investigations undertaken to study the nature and properties of the anti-

bacterial substance produced by the mould, later identified as *Penicillium notatum*, were the genesis of a revolution in the therapy of bacterial infections, and the introduction into medical parlance of a new word—antibiotics. Fleming's first paper on the subject was published in the *British Journal of Experimental Pathology* (1929*b*). In this he recorded the results of his investigations on the antibacterial substance, to which he gave the name 'penicillin'; the temperature range of production of penicillin, its solubility, resistance to heat, filterability, rate of production and stability were accurately described, and a measurement was made of the degree of susceptibility of almost all the known bacteria pathogenic to man, and commensal organisms found in man. Penicillin was shown to possess bacteriostatic, bactericidal and bacteriolytic powers, and the lack of toxicity of the crude penicillin-containing filtrate for animals by intravenous injection, for man by irrigation of infected surfaces and for leucocytes by *in vitro* experiments, was demonstrated. Another fundamental property of even crude penicillin, pointed out by Fleming, was that in contradistinction to all antiseptics its action on bacteria was not neutralized, inhibited or weakened in the presence of blood serum, pus or other exudate. In the original paper (1929) he stated: 'It may be an efficient antiseptic for application to, or injection into, areas infected with penicillin-sensitive microbes', and again in 1931 in an article on some problems in the use of antiseptics: 'It is quite likely that it (penicillin) or a chemical of a similar nature will be used in the treatment of septic wounds.' Unfortunately the instability of the crude penicillin and the small number of septic cases in hospital in peace time, led to its clinical use not being seriously pursued.

Although the use of penicillin for practical therapeutic purposes remained in abeyance till the Oxford workers, Florey and Chain and their colleagues, started their investigations, Fleming used penicillin constantly in the laboratory for selective culture. One of the practical laboratory uses recommended for penicillin was as an aid to the isolation of *Haemophilus influenzae*, which was 'penicillin-insensitive', from nasal and throat swabs and sputum where its presence was often masked or its growth was inhibited by other organisms. In a later communication (1930), it was shown that by the use of penicillin on blood agar plates, *H. influenzae* could be isolated regularly from the gums, tonsils and post-nasal spaces of healthy subjects. The separate and combined uses of penicillin and potassium tellurite for the selective isolation on solid media of organisms from mixed cultures, covering some twenty-six species, and for the demonstration of some bacterial antagonisms, were the subject of a paper in 1932. Subsequently (1942*a*) he demonstrated the value of combinations of penicillin, potassium tellurite and gentian violet in media for differential culture, following Garrod's description (1942) of the selective value of gentian violet for the isolation of *Streptococcus pyogenes*. Maclean (1937), a colleague of Fleming, also used penicillin in a similar manner with great success for the isolation of *Haemophilus pertussis* on cough plates and from swabs. Incorporated in glucose broth penicillin proved invaluable for the isolation of the acne bacillus from pus which contained both the acne bacillus and staphylococcus (Craddock, 1942).

In the meantime, Prontosil, a new chemotherapeutic agent in the treatment of infection, and the first of the sulphonamide drugs, had proved successful in the treatment of infections due to *Streptococcus pyogenes*, meningococcus and gonococcus. Fleming turned his attention to the study by *in vitro* methods of the mode of action of sulphanilamide and the later derivatives, sulphapyridine and sulphathiazole. Although the mode of action remained unsettled, he showed that the action of the sulphonamide drugs was bacteriostatic rather than bactericidal, and that the numbers of infecting organisms, sensitive to the drug, had an important bearing on the ability of the drug to act on them, thus confirming the work of Colebrook and others. He stressed the importance of the bacteriostatic action of the drugs, which allowed the natural defences of the body to deal with the most virulent infections (1940*a*). He also advocated vaccine therapy as an adjuvant to sulphonamide therapy, with a view to increasing the specific immunity (1939).

With the outbreak of the second World War in 1939, interest was again focused on the antiseptic treatment of wounds, and several workers, including Fleming, Dorothy Russell and Garrod, investigated the newer methods of treatment. The older methods of treatment used in the first World War were quickly discarded and investigations centred on the use of sulphonamides and acridine compounds. Fleming, a confirmed antagonist of the use of the older antiseptics, began to find virtue in the use of the sulphonamides and acridines, and although he was not hopeful about the application of sulphonamides as a dressing in septic wounds, with the advent of sulphathiazole he admitted that 'because of its potency and its wide range of activity it would seem that sulphathiazole should be the most effective of the sulphonamide drugs for application to an infected wound with the object of inhibiting the infection until the wound can be surgically cleansed' (1940*b*). He also agreed that the acridine compounds might have equal value, although he had reservations about their possible damage to tissues, a view which was not universally accepted.

In 1941, the Oxford workers, led by Florey and Chain, published a full account of their first investigations on penicillin, and Fleming (1942*b*), using some of the impure solid penicillin powder, supplied by Chain, described *in vitro* methods of testing its potency against *Staphylococcus aureus* and *Streptococcus pyogenes*, using the slide-cell technique for assaying the bacteriostatic power of penicillin in blood. By a comparison of the action of sulphathiazole and sulphapyridine with that of penicillin, which even then had a purity of only 5%, he showed that the latter was eight times more potent against *Staphylococcus aureus* in blood than sulphathiazole and thirty-two times more potent than sulphapyridine. In 1943 he had his first experience of treating a patient with penicillin; the case was one of streptococcal meningitis, and was the first case in which penicillin was administered intrathecally. The rapid and unexpected cure of an almost moribund patient stimulated Fleming to bring penicillin to the notice of the then Minister of Supply, Sir Andrew Duncan; this led to the setting up of the Penicillin Committee, which became instrumental in stimulating the British production of penicillin on

a commercial scale, and encouraging the investigation of methods for its purification, standardization and assay.

Interest in penicillin now became almost world-wide, and the success of trials in treating war wounds during the North African campaign opened up a new vista for research workers in the field and in the laboratory. Fleming, although he was inevitably drawn more and more from the laboratory to deliver lectures and to receive honours which began to be showered on him, yet found time to investigate micromethods of estimating penicillin in blood serum (1944), the penicillin content of blood serum after various doses of penicillin administered by different routes (1947), and the influence of penicillin on blood coagulation (Fleming & Fish, 1947). With May & Voureka (1947) he described a method of titration of streptomycin in patients' serum, and showed that the determination of the end-point depends on the culture medium used, the nature of the test organism, the size of inoculum of the test organism and the presence or absence of oxygen. He also made use of the development of phase-contrast microscopy to demonstrate the extraordinary variations in morphology developed by *Proteus vulgaris* when grown on penicillin agar on microscope slides; by applying intermittent radiant heat to the cultures and studying its effect on motility in the penicillin-induced forms he produced evidence to support the traditional theory of flagella being regarded as the organs of motility in bacteria, contrary to Pijper's claim that bacterial motility was due to gyratory undulating movements of the bacillary body, and that flagella were 'protoplasmic twirls' thrown off from the surface of the organism (1950*a, b*).

Fleming's last published work appeared in 1951, when he advocated with experimental evidence the efficacy of liquid paraffin at a temperature of 130° C. for the sterilization of syringe needles after immersion for 10 sec., a method which had been used effectively by Wright, Fleming and their colleagues for nearly fifty years.

During his medical career of 49 years, Fleming devoted himself to laboratory research on bacteriology and immunology, to teaching and to the application of new knowledge to the prophylaxis and therapy of infective disease. At the time of his death he had more than ninety scientific publications to his name, all based on original work and many of them being descriptions of new methods and techniques which he had evolved. He was also the instigator of many research problems investigated and published by junior members of the staff of the Institute.

There is no doubt that Fleming's choice of career in medicine and his development in the chosen specialty of immunology were largely due to his teacher and friend, Sir Almroth Wright. Wright, like Fleming in later life, did not suffer fools gladly, and no one, as the writer knows, was immune from his incisive and penetrating criticism, but he was a stimulating and inspiring teacher, and Fleming, like many others, owed much to him. Fleming proved an apt pupil at mastering the techniques of the teat, capillary glass tube and slide cell, and soon became a master technician of the art, an art which he retained to the end, because he remained his own technician. He showed great

ingenuity in devising apparatus from the simplest materials, often for the purpose of carrying out an experiment to prove or disprove some immunological idea. His technique was a joy to watch and many of the methods used by Wright and his team owed their origin or perfection to his deft hands. Along with the intellectual ability and phenomenal memory he had shown during his undergraduate career, he developed an originality of thought; this was outstandingly shown round the tea table in the Inoculation Department library, when Wright would raise some immunological problem or propound a theory, and a discussion would follow. Time and again the discussion would finally resolve itself into an argument in which Fleming, the 'little man' as Wright affectionately called him, pitted his mind against the logical and keen insight of 'the old man'. Then, the issue undecided, each would go off to devise an experiment to see which was right. He was possessed of an insatiable curiosity to find out the why and the wherefore of anything that was unusual or new, not only in his work at the bench but also in everyday life; for example, the observation of the stereotropism of leucocytes in fresh blood led him to try the effect of passing blood under the pressure of a rubber teat through the cotton-wool plug of a sterile Pasteur pipette, and he found that this simple procedure removed the leucocytes and platelets from the blood. This in turn led to experiments on the bactericidal power of de-leucocytized blood and further study of the functions of phagocytes *vis-à-vis* staphylococci. The main theme which runs through all Fleming's work is the study of the natural defences of the body against infection, in particular the antibacterial powers of the phagocyte and the blood serum and their interrelated functions. During the first World War and after, he became one of the antagonists in chief against the use of antiseptics for the treatment of wounds or systemic infections, and much of his research was devoted to proving the adverse effect of antiseptics on the natural defences of the body, a subject which he pursued with a missionary zeal and on which he was at times outspoken.

His discoveries of lysozyme and penicillin are two outstanding examples of his perceptive powers and curiosity to investigate the unusual. He made no secret of the fact that the discovery of lysozyme gave him more pleasure than any of his other work, not excluding the discovery of penicillin which was to make his name a household word. Lysozyme, later shown to be an acetylamino polysaccharidase, is the only one of the 'cytases' postulated by Metchnikoff, so far shown to be able to cause lysis of living bacteria, and subsequent studies have confirmed Fleming's views of its importance as a factor in natural immunity.

Surprise has been expressed in some scientific circles that Fleming did not pursue further his investigations on lysozyme and penicillin, especially the latter, but it must be remembered that Fleming was a bacteriologist and immunologist, not a chemist, and there was no chemist available to co-operate with him. As he himself pointed out, even skilled chemists had failed in their attempts to concentrate, stabilize and purify penicillin, before success was eventually achieved through the joint efforts of a team of workers, each a specialist in his own field. Although, working unaided, he was not able to

pursue his investigations of penicillin to their ultimate fulfilment, tribute must be paid to his views expressed in 1929 and again in 1931 that penicillin had a future for the treatment of infection, and to his initiative in persuading the Ministry of Supply and Ministry of Health of the importance of penicillin in the war effort. Great technical advances in scientific knowledge and its application occur in the stress of war, and penicillin was discovered at a time unpropitious from this point of view. Again, team work in medical science is a comparatively recent development, especially in the field of the chemistry of bacterial metabolism, and without such team work, both in England and U.S.A., the pursuit of penicillin to its full fruition might still be 'a consummation devoutly to be wish'd'. Although his main interest was immunity and infection, a list of his published work shows that Fleming carried out a considerable amount of research on current problems, in particular the development of selective media for differential culture of the common pathogenic bacteria, the uses of nigrosin, alone or combined with stains, as an excellent method of demonstrating bacterial morphology and spores, and a simple and rapid technique for staining bacterial flagella, used with success in the teaching of students.

In 1948 Fleming retired as Professor of Bacteriology with the title Emeritus but continued as Director of the Wright-Fleming Institute of Microbiology until his death, although he had decided shortly before to hand over the reins to his successor, Professor R. Cruickshank. After the establishment of penicillin as a therapeutic agent of the highest potency against infection, it was inevitable that Fleming should be drawn from his laboratory to deliver lectures and receive the honours accorded him by universities, societies, cities and nations throughout the civilized world, as a tribute to his work. He received the Fellowship of the Royal Society in 1943, of the Royal College of Physicians of London in 1944, and of the Royal College of Physicians of Edinburgh in 1946. In 1945 the Nobel Prize for Medicine was awarded jointly to the three men most concerned in the discovery, purification and application of penicillin for therapeutic use—Fleming, Florey and Chain. Fleming was knighted in 1944, and was the first foreign citizen to receive the United States Medal of Merit. In the years that followed, the honorary degree of Doctor of Science was conferred on him by six European and three American Universities; the honorary degree of Doctor of Medicine by seven European Universities, and the honorary degree of Doctor of Laws by two Scottish Universities. Medals and prizes awarded to him included the Gold Medal Royal Society of Medicine, Hon. Gold Medal Royal College of Surgeons of England, the Moxon Medal Royal College of Physicians, the Harben Gold Medal Royal Institute of Public Health and Hygiene, the Albert Gold Medal Royal Society of Arts, the Medal for Therapeutics of the Society of Apothecaries, the John Scott Medal City Guild of Philadelphia, the Actonian Prize Royal Institution, and the Cameron Prize University of Edinburgh. He was William Julius Mickle Fellow of London University in 1942, and Charles Mickle Fellow of Toronto University in 1944. He was Linacre Lecturer to the University of Cambridge, Harben Lecturer to the Royal Institute of Public

Health and Hygiene, Cutter Lecturer to Harvard University, Mayo Foundation Lecturer to the University of Minnesota, Lister Memorial Lecturer Society of Chemical Industry, Robert Campbell Lecturer Ulster Medical Society, and Shattuck Lecturer Massachusetts Medical Society, among others. He was made a Fellow or Honorary Member of many Academies of Medicine and Science, including the French Academy of Science, the Pontifical Academy of Sciences, and the Royal Society of Copenhagen. He had been President of the Society for General Microbiology, and of the Sections of Pathology and Comparative Medicine, Royal Society of Medicine. From 1945 to 1949 he was a member of the Medical Research Council and in 1946 he became chairman of a committee set up to organize clinical trials of streptomycin and other antibiotics in non-tuberculous conditions. In 1951 he was elected Rector of Edinburgh University. He was Honorary Freeman of Darvel, Chelsea and Paddington, the Boroughs where he was born, lived and worked; in 1949 he was made an Honorary Freeman of the City of Athens, and of the City of Verona in Italy on the occasion of the International Medical Congress. He was made Commander of the Legion of Honour in France, Honorary Chief of the Kiowa Tribe, and received the Grand Cross of Alphonse X The Wise, in Spain. Shortly before his death he had completed plans for a lecture tour in the Middle East on behalf of the British Council.

Fleming, the man, was short, broad-shouldered and deep chested, and his eyes were keen and expressive. During 34 years as pupil and friend, the writer never saw him lose his temper or speak ill of anyone, although on the rare occasions when he was annoyed his eyes could flash fire, and a look was more expressive than the spoken word. Although he was not a great lecturer, he had the gift of lucid exposition, and made up in sincerity what he lacked in eloquence. He has been described as taciturn and laconic, but he was a good listener, quick to grasp the essentials of an argument or discussion and give the *coup de grâce* to any ill-conceived theory. In informal discussions he delighted to take views opposite to those expressed, although he may have secretly agreed with them, and in this way he often extracted valuable ideas for experimental work; he used this technique to enable him to assess the originality and agility of mind of young workers, and to encourage in them clear thinking and reasoned argument. His phenomenal memory and quick mind served him well in reading the journals; his reading was done in short spells, as he could not remain inactive for more than half an hour or so, whether in the laboratory, at home, or in the country. He was a strong and loyal supporter of St Mary's Hospital Medical School, and was always ready to undertake any work to advance its interests and help the students in all their activities, in the class, in the ward and in the field of sport. Although he was fond of people and company and made many friends in different walks of life, he was not easy to know, due perhaps to his modesty and diffidence. To his close friends and intimates he was 'Flem', and only to the few was he 'Alec'. He was an excellent host and entertained much, both at his flat in Chelsea and his country home at Barton Mills in Suffolk. He had a keen artistic sense and was no mean performer with water colours. His handwriting was small

and copperplate, and the beautiful neat draughtsmanship of his lantern slides and innumerable diagrams in black and white and in colour, prepared both for teaching purposes and for illustrations in scientific papers, will be treasured in the Wright-Fleming Institute. He was for many years an honorary member of the Chelsea Arts Club, and numbered many artists among his friends—he was indeed the unofficial medical consultant to the club. He was a keen and expert photographer and applied his skill in this art both in the laboratory and as a leisure occupation, with excellent results. In his younger days he was a fine rifle-shot and a good swimmer, but in middle life he played a vigorous game of billiards, and could defeat younger and more experienced players at tennis, golf and croquet. Of all his leisure activities there is no doubt that gardening gave him the greatest pleasure—fruit, flowers and vegetables grew in profusion at his country residence at Barton Mills; he had ‘green fingers’ and was forever experimenting with cuttings, cross-pollination, new plants or seeds and grafting—much of the produce of his garden went to the wards of St Mary’s Hospital. He loved to browse in second-hand bookshops and was specially interested in history and colour drawings of birds and insects. He was a collector of old silver and glass and had some fine examples of Georgian silver and old English cut glass.

In 1921, the year in which he discovered lysozyme, he married Sarah Marion McElroy of County Mayo, who shared many of his interests, especially love of gardening and the collection of antiques. She died in 1949, leaving one son Dr Robert Fleming. In 1958 he married Dr Amalia Coutsouris from Greece, who had come to work on the staff of the Wright-Fleming Institute in 1947, and had returned to Greece in 1952 to become head of the bacteriological department of a hospital in Athens. During the war she commanded respect by helping British soldiers during the Greek campaign, for which she was imprisoned by the Germans.

Fleming remained active and interested in his work to the last, and he had planned in retirement to set up a laboratory, where he could continue his work, in a studio in the grounds of his country home. It was fitting that at the end his ashes were interred in St Paul’s. He had served his day and generation well and had been instrumental in saving more human lives and suffering than anyone in the history of medicine. His name will surely be remembered for evermore.

V. D. ALLISON

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The Isolation and Chemical Nature of Capsular and Cell-wall Haptens in a *Bacillus* Species

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SUMMARY: By extracting the capsular material of *Bacillus* M with hot water, D-glutamic acid polypeptide and a polysaccharide were isolated. The polypeptide absorbs the antibody from an anthrax immune serum, which reveals a homogenously distributed capsular material in *Bacillus* M. The polysaccharide absorbs one of the homologous antibodies, which renders a characteristic structure visible in the capsule and gives a specific cell-wall reaction. By using lysozyme digestion for the dissolution of the capsule or of the cell-wall a mucoprotein was isolated. The mucoprotein gives serological reactions identical with those of the polysaccharide, and it corresponds to a less degraded form of the cellular substance participating in building up both the cell wall and the capsular structure.

We have shown that a morphologically and chemically complex structure can be demonstrated, by the use of appropriate antibodies, in the capsule of *Bacillus megaterium* and the related *Bacillus* M (Tomesik, 1951; Tomesik & Guex-Holzer, 1951, 1954*b*, *c*). Anthrax D-glutamic polypeptide antibody made visible homogenously distributed material in phase-contrast. The homologous immune serum revealed polar bodies and conspicuous transverse septa in the capsule. It was shown at the initial stage of lysozyme digestion that the capsular transverse septa appeared as a direct continuation of the cross cell walls and that the homologous serum also enhanced the visibility of the cell wall. A similar specific cell-wall reaction was also demonstrated by adding the same serum to trypsin-treated or to autolysed bacteria. Our previous work indicated that the antibody reacting with both the capsular transverse septa and the cell wall can be absorbed by material composed mainly of polysaccharide. The purpose of the present paper is to describe two methods for the isolation of a polysaccharide and of a mucoprotein. These substances contain an immunologically active component and appear to absorb the antibody responsible for both reactions.

The capsular material was extracted simply by boiling an aqueous suspension of the bacteria. A similar method was used for the extraction of polysaccharides by Webb (1948) working with *Staphylococcus citreus*, and by Aubert (1949) with capsulated *Bacillus megaterium*. In the hot-water extract of capsulated *Bacillus* M both D-glutamic acid polypeptide and a polysaccharide were found. For the separation of these two substances a method different from those described previously (Tomesik & Szongott, 1933; Ivanovics & Brückner, 1937; Hanby & Rydon, 1946) was used.

The extraction of serologically active material from non-capsulated *Bacillus* M was extremely difficult. Alkalies, acids, sodium taurocholate,

phenol, formamide or antiformin were tried without success; extracts exhibited only slight serological activity. Previous observations in the literature suggested to us the use of lysozyme for extraction. We found (Tomesik & Guex-Holzer, 1952) with phase-contrast that lysozyme dissolved the cell walls and the capsular structures of *Bacillus M* within a short time without destroying the cytoplasmic membrane. Salton (1952a) reported, simultaneously, that lysozyme dissolved the mechanically separated cell walls of *Micrococcus lysodeikticus*.

METHODS

Preparation of organisms. The strain of *Bacillus M* used in this work invariably grew on Gladstone & Fildes CCY agar in capsulated form without the addition of glucose. Kolle flasks containing 70 ml. agar were inoculated from a 12 hr. agar culture. The flasks were incubated for 20 hr. at 32°; the organisms were suspended in tap water and the suspension filtered through gauze. The average dry weight of organisms from one Kolle flask was 0.27 g. Non-capsulated bacilli were obtained in Gladstone & Fildes liquid medium aerated for 24–48 hr. at 30°. The growth from liquid medium was washed twice with tap water in a Sharples centrifuge.

Cell-wall preparations were made by digesting a living suspension of bacteria with trypsin (Siegfried, Zofingen), 5 mg./ml. at 37° for 15 hr.

Immune sera. An immune serum produced by fifteen intravenous injections of heat-killed capsulated *Bacillus anthracis* in rabbits is referred to in this work as 'polypeptide antibody', since only the D-glutamic acid polypeptide antibody of the anthrax serum reacted with *Bacillus M*. The homologous antibody was produced by ten injections of living *Bacillus M* suspensions in rabbits.

Specific capsule and cell-wall reactions were carried out with immune sera as described previously (Tomesik & Guex-Holzer, 1954b).

Precipitin reaction was performed in capillary tubes by layering serial dilutions of extract on undiluted immune serum. This controlled the efficiency of the major steps in the fractionation.

Hydrolysis. The glutamic acid polypeptide was hydrolysed with 6N-HCl under reflux for 15 hr. The mucoprotein and the polysaccharide were hydrolysed with 2N-HCl for 1.5 hr. for determination of reducing substances and with 6N-HCl for 15 hr. for paper chromatography.

Paper chromatography. Phenol and collidine were used as solvents. The spray reagents were ninhydrin for amino acids and amino sugars, *p*-dimethyl-amino-benzaldehyde for amino sugars, aniline hydrogen phthalate for reducing and for some non-reducing sugars and naphthoresorcinol for non-reducing sugars. The hydrolysate was placed on Whatman filter-paper no. 1. The method used in two-dimensional chromatograms to compare the R_F value of an unknown substance with that of the standard substance was the following: when, for example, the presence of glucosamine had to be tested, the hydrolysate was deposited on the left lower corner of a filter-paper, whereas the standard glucosamine solution was applied at the right lower and left upper corners. The paper was treated in one direction with phenol, in another

with collidine, then sprayed. Straight lines drawn through the spots of the standard substance indicated at their intersection the place where the spot of an identical substance of the hydrolysate should be located. With this method we could differentiate the control spots of glucosamine and galactosamine.

Total nitrogen was estimated by the method of Koch & McMeekin (1924), *phosphorus* by the method of Fiske & Subbarow (1925) and *reducing sugars* by the method of Folin & Wu (1920); *reducing power* was expressed as glucose. The colorimetric readings for these estimations were performed in a Klett-Summerson colorimeter.

Acetyl estimation was by the method of Freudenberg & Weber (1925).

Lysozyme was prepared from egg-white following the procedure of Alderton & Fevold (1946). For comparison a purified lysozyme preparation of Mann Research Laboratories was used.

RESULTS

Hot-water extraction of capsulated bacteria

Capsulated bacteria (dry weight 14 g.) were suspended in 1200 ml. tap water, shaken with 10 ml. chloroform and kept at room temperature overnight. The capsules seemed to be intact and showed a large volume in indian ink preparation (Pl. 1, fig. 1). The suspension was then heated in a bath of boiling water for 4 hr. During this procedure the capsules became successively thinner (Pl. 1, figs. 2, 3) but they were detected even after 4 hr. in c. 50 % of the bacteria. More prolonged heating did not result in appreciable further extraction. The bacteria were then centrifuged down and washed twice. The mixed yellowish brown, somewhat turbid supernatants (1100 ml.) were concentrated at 60° in vacuo to 200 ml., centrifuged and the almost clear supernatant precipitated with 500 ml. 96 % (v/v) ethanol in water. (When not stated otherwise ethanol means 96 % (v/v) ethanol in water.)

The weight of the ethanol precipitate was 3.5 g. (25 % (w/w) of the dried bacteria) and when dissolved in 100 ml. water, the resulting solution was somewhat turbid, very viscous and gave a strong precipitation reaction with the polypeptide antiserum as well as with the homologous immune serum. By addition of 0.7 vol. ethanol an almost inactive substance was brought down and discarded. A rough separation of two substances giving different serological reactions was attained by fractional precipitation with ethanol; 1.1 vol. ethanol brought down the bulk of the substance (fraction A) which reacted with polypeptide antibody; 2.5 vol. ethanol precipitated fraction B which reacted mainly with the homologous antibody.

Isolation of D-glutamic acid polypeptide from fraction A

Among the substances contained in fraction A were inactive proteins and some bacteria which were not separated during centrifugation of the strongly viscous solution. Inactive proteins were removed by dialysis after digestion with trypsin. Trypsin also digested the bacteria with the exception of the cell walls and some other residual substances which were precipitated with barium acetate.

Fraction A was dissolved in 130 ml. 0.85 % NaCl and digested with 20 mg. trypsin (pH 8) at 37° for 24 hr. To prevent contamination 3.5 ml. chloroform was added and the pH value readjusted from time to time with NaOH. Barium acetate (200 mg.) was then added and the solution was rapidly heated to 100°. The coagulated material, including trypsin, was centrifuged down. The digested proteins were removed by dialysis for 2 days. The polypeptide was precipitated from 150 ml. dialysed solution by the addition of sodium acetate and 120 ml. ethanol. This procedure was repeated twice and the precipitate freeze-dried after 2 days dialysis in distilled water. A total of 685 mg. of readily water soluble polypeptide material was obtained, amounting to 4.5 % (w/w) of the bacteria.

Isolation of the capsular polysaccharide from fraction B

The serologically inactive substances of fraction B which were difficult to remove by the usual methods of purification, formed insoluble compounds with several proteins at acid reaction. We assumed that this reaction might be similar to the one we described between capsular substances and proteins (Tomsik & Guex-Holzer, 1954a), being probably elicited at a pH value lying between the isoelectric points of the two reacting substances. Indeed it was observed that the glutamic acid polypeptide, an acidic substance, was precipitated by bovine albumin at pH 3-4.5, when the basic groups of albumin were dissociated. In eliminating the impurities of fraction B we used bovine plasma albumin fraction V (Armour and Co. Ltd., London) at an appropriate pH value.

Fraction B was dissolved in 100 ml. McIlvain's buffer solution (pH 3.2), and while shaking 5 % (w/v) albumin was added until maximum turbidity was obtained. The resulting granular precipitate was easily removed in the centrifuge, and, when dissolved by adjusting to pH 7, gave no reaction with the homologous immune serum.

It was occasionally observed that the precipitate obtained after the addition of protein at pH 3.2 could not be fully separated. However, by altering the pH in an appropriate direction flocculation of the turbid suspension could be effected. Different impurities probably require different pH optima to form insoluble compounds with the protein added. It was sometimes necessary to change the pH value several times to eliminate all impurities which reacted with proteins. This method can be used for purification of bacterial extracts only when the substance to be isolated is not involved in the reaction.

The precipitated protein compound was washed with buffer at pH 3.2. The clear colourless supernatant fluids were combined and neutralized with N-NaOH. At pH 4.8, at the isoelectric point of the albumin, the solution became cloudy. When 450 ml. ethanol were added to the 250 ml. solution the rest of the albumin was largely precipitated. After addition of 750 ml. ethanol a polysaccharide containing a serologically active fraction was precipitated. This fraction was deproteinized by Sevag's method (1951) and after ethanol fractionation was dissolved in formamide. From the formamide solution a small amount of inactive substance was precipitated by ethanol. The polysaccharide was dissolved in distilled water and precipitated with

0.5 vol. ethanol without the addition of sodium acetate. This procedure was repeated and the substance freeze-dried. Dialysis was omitted since in previous experiments it led to loss of material. Polysaccharide (71 mg.) was obtained as a white powder, easily soluble in water.

Extraction of capsulated bacteria with lysozyme and isolation of a mucoprotein

Capsulated bacteria (15 g.) were suspended in 70 ml. 0.85% NaCl. The suspension was placed in a 60° water-bath for 30 min.; lysozyme (4 mg.) was then added, and after shaking the mixture was incubated at 45°. The suspension was examined frequently by phase-contrast microscope for dissolution of capsules. After about 30 min. the capsules were no longer visible. To prevent a visible dissolution of the cell walls, the suspension was immediately chilled and spun in a Sharples centrifuge. The supernatant was deproteinized according to Sevag's method. After drying in vacuo at 60° 1.5 g. of material was recovered which gave a clear solution in 200 ml. 0.85% NaCl. It gave equally strong serological reactions with the homologous immune serum as well as with polypeptide antibody. The polypeptide was eliminated by fractional ethanol precipitation as described previously (see hot-water extraction procedure). Further purification of the mucoprotein fraction was carried out by precipitating the substance with 96% ethanol saturated with sodium acetate, first from formamide solution, then from solution in saturated urea. When by a repetition of these procedures no more serologically-inactive substances were removed, the distilled water solution of the material was dialysed and freeze-dried.

Mucoprotein (340 mg.) in the form of a white powder very easily soluble in water was thus obtained from the lysozyme extract of capsulated bacteria. The serological activity of this material with the homologous immune serum was about the same as that of the polysaccharide purified after hot-water extraction. It did not react with polypeptide antibody. It should be pointed out that by the lysozyme method about five times more serologically active material could be obtained than by hot-water extraction.

Extraction of non-capsulated bacteria with lysozyme and isolation of a cell-wall mucoprotein

As shown previously (Tomcsik & Guex-Holzer, 1952; Salton, 1952a) the cell wall of *Bacillus M* can also be dissolved within a short time when an appropriate concentration of lysozyme is applied. Several batches of bacteria were treated with lysozyme for the isolation of purified cell-wall mucoprotein. The method of isolation is illustrated here with one example.

Non-capsulated bacteria (2 g.) were suspended in 40 ml. 0.85% NaCl and placed for 30 min. in a 60° water-bath. The suspension was then chilled, 5 mg. lysozyme added and shaken without glass beads for 15 min. in a Mickel vibrator to ensure a homogenous distribution and the absorption of lysozyme. Free lysozyme was removed by centrifugation. The bacteria treated with

lysozyme treated bacteria were suspended in 50 ml. 0.85% NaCl and placed in a 35° water-bath for 2 hr.

At this stage phase-contrast microscopy revealed only partially degraded 'small bacteria' but no cell walls. To prevent a further disintegration of mucoprotein, the suspension was chilled and centrifuged. The sediment was washed twice in 0.85% NaCl, after which it was shaken for a short time in a Mickle vibrator to facilitate resuspension.

The washing fluids and extract were mixed and fractionated further with essentially the same procedure as used for the purification of the capsular mucoprotein. Slight modifications were, however, occasionally necessary, probably owing to the different substances extracted from the cell wall. Fifty mg. mucoprotein were obtained (2.5% (w/w) of the bacteria). The yield was proportionately the same as by the much less intensive lysozyme extraction of capsulated bacteria. The appearance and the solubility of the cell-wall mucoprotein were similar to those of the 'capsular' mucoprotein.

Properties of the isolated substances

The data of the serological properties and chemical analysis of hot-water extracts in different stages of purification are summarized in Table 1. As can be seen the polypeptide of fraction A was precipitated without admixture of polysaccharides by adding 1.1 vol. ethanol to the raw extract. Fraction B

Table 1. *Isolation of D-glutamic acid polypeptide and of a polysaccharide by hot-water extraction of capsulated bacilli*

Fraction	Weight (mg.)	Precipitin titre		N (%)	P (%)	Reducing substance (%)
		Polypeptide antibody	Homologous serum*			
Raw extract	3550	5×10^5	1×10^5	4.3	6.3	n.t.
Fraction A	1375	1×10^6	—	6.9	5.3	n.t.
Fraction B	1150	5×10^4	2×10^5	2.6	8	n.t.
Polypeptide from fraction A	685	2×10^6	—	10	—	—
Polysaccharide from fraction B	71	—	2×10^6	4	0.5	40

— = negative; n.t. = not tested.

* Antiserum to capsulated *Bacillus M.*

contained, however, apart from the polysaccharide, an appreciable amount of polypeptide as well as serologically inactive substances. These substances could be eliminated during the course of subsequent fractionation with partial loss of the polysaccharide fraction.

The properties of the mucoprotein obtained with lysozyme extraction of capsulated bacteria are given in Table 2. Serological and chemical analysis of the capsular and cell-wall mucoproteins revealed no essential difference between the two substances. Both of them failed to give precipitation with anthrax polypeptide antibody, and apparently they precipitated the same

antibody of the homologous immune serum. Furthermore their serological reactions were identical with those of the polysaccharide. It should, however, be noted that whereas the precipitin titre of the polysaccharide isolated from the hot-water extract of capsulated bacteria remained stable, the titre obtained with the mucoproteins started to decrease within a few days of their isolation. Within 1 or 2 months the serological activity of the mucoproteins, isolated after enzymic digestion of the capsule or of the cell wall, decreased to at least one-tenth of the original value even when they were kept at 4° after freeze-drying.

Table 2. *Characteristics of the capsular and cell-wall mucoprotein*

Substance	Initial weight of bacteria (g.)	Weight of purified substance (mg.)	Precipitin titre with homologous serum	N (%)	P (%)	Reducing substance (%)	Acetyl (%)
Capsular mucoprotein	15	340	1×10^6	7.8	4.1	22.5	4.3
Cell-wall mucoprotein	2	50	1×10^6	7.6	4.7	18	n.t.

n.t. = not tested.

Further information about the chemical nature of the isolated haptens after their hydrolysis (see Methods) was obtained through paper chromatography.

Only glutamic acid was found in our capsular polypeptide preparation when smaller amounts of hydrolysate were placed on the paper. However, by depositing 50–100 µg. of the hydrolysed polypeptide a second faint spot appeared after ninhydrin spraying, corresponding to no. 6 on Fig. 2.

The result of analysis of the capsular polysaccharide is shown in the two-dimensional chromatogram after spraying with *p*-dimethylaminobenzaldehyde in Fig. 1. Galactosamine and glucosamine were identified by using standard substances for control. The spot no. 3 was very weak. It reacted like an amino sugar with ninhydrin, with aniline hydrogen phthalate and with *p*-dimethylaminobenzaldehyde. It showed, however, no reducing power when tested in the following way:

Equal sized paper strips of the collidine chromatogram were cut out, each containing a separate spot and were extracted. Extracts of glucosamine showed reducing power, the extract of spot no. 3 did not.

Hot-water extraction and chromatographic analysis of the purified polysaccharide were carried out several times. Results showed a variation between the ratio of the glucosamine to galactosamine, probably in accordance with slight variation in the synthesizing capacity of the strain. During the first period glucosamine, later galactosamine predominated in the hydrolysed polysaccharide. This difference is noted while admitting that the differentiation of glucosamine and galactosamine by two-dimensional chromatography with phenol and collidine solvents does not give an assured means of identification.

Chromatographic analysis of the capsular and cell-wall mucoprotein showed the same results independent of their source. Results obtained in the two-

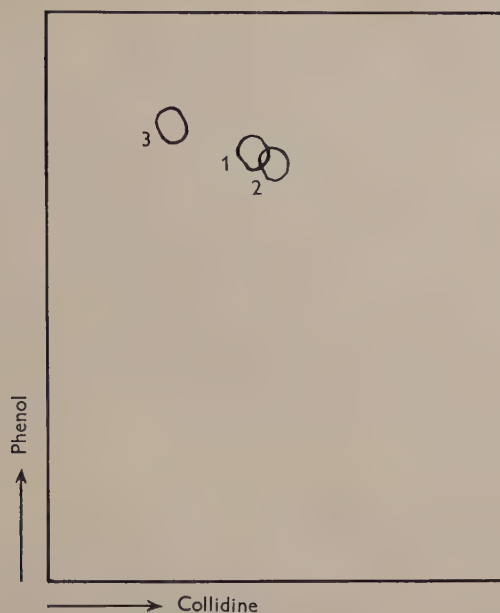


Fig. 1. Chromatogram of hydrolysed capsular polysaccharide sprayed with *p*-dimethylaminobenzaldehyde. 1, galactosamine; 2, glucosamine; 3, see text.

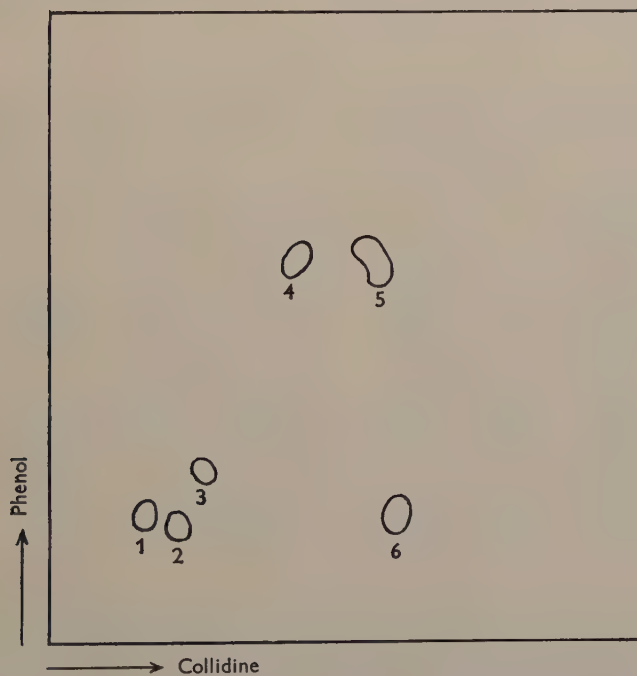


Fig. 2. Chromatogram of hydrolysed mucoprotein sprayed with ninhydrin. 1, diamino-pimelic acid; 3, glutamic acid; 4, alanine; 5, hexosamine; 2, 6, see text.

dimensional chromatogram are shown in Fig. 2. Hexosamine was found constantly in all of the mucoprotein hydrolysates but sometimes glucosamine, at other times galactosamine, seemed to predominate. Alanine occurred also in all of the preparations but its spots were of varying intensity. Glutamic acid spots were always very pale. Presence of diaminopimelic acid was assumed only on the basis of R_F value; it was, however, not controlled with a standard preparation. The non-identified spot no. 2 reacted strongly with ninhydrin; in a mixed preparation its R_F value was slightly different from aspartic acid; similarly, the non-identified spot no. 6 was found only occasionally in different mucoprotein preparations, but it could be demonstrated regularly in polypeptide preparations when a larger quantity of hydrolysate was placed on the paper. This spot gave a very faint bluish-grey colour with ninhydrin, and it corresponds probably to the unknown hexosamine in spore peptides reported by Strange & Powell (1954).

Reactions of immune sera after absorption with specific substances

It was easily established that the capsular polypeptide did not show any serological relationship, either with the capsular polysaccharide or the mucoproteins. On the other hand, the mucoproteins isolated from capsulated and non-capsulated bacteria were serologically and chemically identical, and they contained the same hapten as the capsular polysaccharide. Since the last-mentioned substances were identical in their direct serological reactions as well as in their capacity of absorption, we have summarized in Table 3 only the polypeptide and polysaccharide absorptions.

Table 3. *Absorption of sera by capsular polypeptide and polysaccharide*

Antibody	Hapten used for absorption	Reaction	Result
Anthrax-polypeptide	—	Visibility of capsule	+
Anthrax-polypeptide	Polypeptide	Visibility of capsule	—
Anthrax-polypeptide	Polysaccharide	Visibility of capsule	+
Homologous serum	—	Visibility of capsular structure	+
Homologous serum	Polypeptide	Visibility of capsular structure	+
Homologous serum	Polysaccharide	Visibility of capsular structure	—
Homologous serum	—	Specific cell-wall reaction	+
Homologous serum	Polypeptide	Specific cell-wall reaction	+
Homologous serum	Polysaccharide	Specific cell-wall reaction	—
Homologous serum	—	Agglutination of living bacteria	1:3840
Homologous serum	Polysaccharide	Agglutination of living bacteria	1:1280
Homologous serum	—	Agglutination of cell-wall preparation	1:960
Homologous serum	Polysaccharide	Agglutination of cell-wall preparation	1:30

Since the specific capsular and cell-wall reactions could not be elicited even with the best immune serum when this was diluted more than 1:30, absorption for these reactions was carried out by mixing equal volumes of the undiluted immune sera and a 1:200 (w/v) dilution of the specific substances.

This mixture contained a large antigen surplus and precipitation was inhibited. The mixtures were kept first 30 min. at 37° and subsequently for 5 hr. at 4°.

As higher dilutions resulted in agglutination 1 vol. of serum was absorbed with 9 vol. of 1:2000 (w/v) dilution of the hapten before carrying out the agglutination tests. No specific capsular reaction was obtained with homologous serum after its absorption with polysaccharide (Pl. 1, fig. 4). The non-absorbed homologous serum, or the same serum after polypeptide treatment, revealed the capsular transverse septa (Pl. 1, fig. 5), whereas successive addition of an anthrax polypeptide antibody made the homogeneously distributed capsular material visible (Pl. 1, fig. 6). The last-mentioned reaction was not influenced by adding polysaccharide to immune serum; it could not, however, be obtained after polypeptide absorption of this serum.

The results of agglutination tests with absorbed sera listed in Table 3 require special comment. The agglutinin titre of homologous serum against living bacteria was but slightly decreased after polysaccharide adsorption, whereas the absorbed serum did not agglutinate the cell-wall preparations in an essentially higher titre than a normal serum. In another communication we have reported that the homologous immune serum contains, apart from the polysaccharide antibody, a protein antibody which reacts in the agglutination test with a thermolabile surface antigen not present in the cell-wall preparations obtained by trypsin digestion of the bacteria (Tomesik & Guex-Holzer, 1954d).

DISCUSSION

We assume that the specific substances isolated and characterized in this work are homogeneous. We base this assumption on the observation that various methods of fractionation did not reveal any further change in serological activity and in the chemical composition of the purified substances. Since capsular polysaccharide and mucoprotein gave an identical serological reaction, we can safely conclude that the basis of their serological specificity is dependent on the special configuration of the substance of simpler chemical composition, i.e. of the polysaccharide. On the other hand, it can be concluded that the mucoprotein resembles more closely the composition of the specific substance as this occurs in the capsule and in the cell wall. Hot-water extraction at neutral reaction apparently splits off the polysaccharide from much more complex molecules within the cell structure. It is also probable that the mucoprotein we isolated after lysozyme extraction is a degradation product and is split off from its combination with ribonucleic acids, as can be surmised from the observation of Webb (1948). The specific polysaccharide can, therefore, be regarded as an identical component of more complex unknown substances forming on the one hand the cell wall and on the other hand the peculiar capsular structure of *Bacillus M*. The D-glutamic acid polypeptide occurs in the capsule but it does not contribute to the composition of the cell wall.

Can the mucoprotein be regarded as the substrate of *Bacillus M* which is acted upon by lysozyme? Before we attempt to answer this question it should be pointed out that our knowledge about the substrate of lysozyme is based on experiments essentially different from those reported in this paper. Meyer, Palmer, Thompson & Khorazo (1936), and Epstein & Chain (1940) isolated polysaccharides from lysozyme-sensitive micro-organisms using methods which disintegrated the whole cellular structure. Since they found that from these polysaccharides one-third of their reducing sugars were liberated after a prolonged lysozyme digestion, they regarded the polysaccharides as the substrate of lysozyme. Feiner, Meyer & Steinberg (1946) confirmed these observations and found that a mucopolysaccharide isolated by alkaline extraction of *Micrococcus lysodeikticus* retained its original serological activity after digestion with lysozyme for 1 hr. The primary purpose of the present work was quite different. It was demonstrated previously that lysozyme acts on the capsular structure and on the cell wall of *Bacillus M* and that both of these cellular elements were made visible in phase-contrast by the same immune serum (Tomcsik & Guex-Holzer, 1952). In the present work a mucoprotein and a polysaccharide were isolated, both of which absorbed the antibody, which rendered these cellular structures visible. These observations might justify the assumption that the mucoprotein corresponds more closely to the cellular substrate acted upon by lysozyme than does the purified polysaccharide. This assumption seems to us probable because the mucoprotein was extracted after lysozyme disintegration of the capsular, i.e. of the cell-wall structure and because its serological reactivity was largely inactivated when kept for 1 or 2 months at 4° after freeze-drying. The natural cellular substance attacked in living bacteria by lysozyme is probably much more complex than the mucoprotein as can be surmised from the excellent studies of Salton (1952b, 1953) on the chemical composition of the isolated bacterial cell wall.

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S. GUEN-HOLZER & J. TOMCSIK—NATURE OF CAPSULAR AND CELL-WALL HAPTENS. PLATE 1

(Facing p. 25)

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EXPLANATION OF PLATE

Capsulated *Bacillus* M by phase-contrast microscopy. All figures at $\times 2000$.

- Fig. 1. Indian ink preparation before extraction.
- Fig. 2. Indian ink preparation after extraction in a bath of boiling water for 2 hr.
- Fig. 3. Indian ink preparation after extraction in a bath of boiling water for 4 hr.
- Fig. 4. A long chain after the addition of homologous serum absorbed with polysaccharide.
- Fig. 5. The same chain as in fig. 4 after the addition of non-absorbed homologous serum.
- Fig. 6. The same chain as in fig. 5 after the successive addition of anthrax polypeptide antibody.

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The Relation of Ribose Nucleic Acid to the Early Stages of Induced Enzyme Synthesis in Yeast

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SUMMARY: The synthesis of deoxyribonucleic acid (DNA) in yeast can be abolished by dosages of ultraviolet light (UV) which permit ribonucleic acid (RNA) and protein synthesis to continue. Those dosages of UV which inhibit α -glucosidase synthesis prevent not only the net utilization of the free amino acid pool but also inhibit glycine incorporation into proteins and decrease to a minimal value glycine and phosphate incorporation into RNA. The latent period before the appearance of α -glucosidase was characterized by an increased sensitivity to irradiation and certain amino acid analogues. The significance of these results in an interpretation of the early stages in enzyme induction is discussed.

Previous investigations on induced α -glucosidase synthesis in yeast led to the conclusion that free amino acids were the precursors of the enzyme and that the first stable intermediate formed must be of sufficient complexity to involve a large percentage of these amino acids (Halvorson & Spiegelman, 1952; Halvorson, Spiegelman & Hinman 1955). Over the past four years a continued attempt to identify peptide intermediates by a wide variety of techniques and under various conditions had failed. We therefore accepted as a working hypothesis a template-type mechanism for the enzyme-forming system. Unfortunately such a conclusion almost leads to an impasse in attempts to design experiments to study the mechanism of enzyme synthesis. Although experiments with radioactive amino acids have proved valuable in studying protein synthesis, they have thus far given little information on the mechanism involved.

At present, it would seem profitable first to gain further information on the nature of the enzyme-forming system (EFS) itself. Two approaches for providing such information are as follows. First, the dissociation of enzyme synthesis from non-essential cellular functions. The recent separation in yeast of the EFS from the processes concerned with cell viability (Barron, Spiegelman & Quaster, 1953), and the demonstration of enzyme synthesis in disrupted bacterial cells (Gale, 1955) illustrate the usefulness of this approach. The second approach involves an investigation of the early stages of induced enzyme synthesis. The induction of penicillinase in *Bacillus cereus*, which involves several distinct stages (Pollock, 1953), and the observation by Borsook (1955) that the rate of enzyme synthesis is actually very slow compared to enzymic reactions suggest the possibility of isolating stages in the synthesis of an enzyme molecule. The purpose of the present paper is to furnish additional information (Halvorson & Jackson, 1954), by these two approaches, on the nature of the EFS in α -glucosidase synthesis in yeast.

METHODS

A diploid representative of *Saccharomyces cerevisiae*, strain LK2G12, was grown in standing flasks at 30° in a complete medium prepared by adding the following to 1 l. of water: Bacto-Peptone, 5 g.; yeast extract, 2.5 g.; 60% (w/v) sodium lactate, 6.0 ml.; CaCl₂, 0.25 g.; MgSO₄·7H₂O, 0.25 g.; KH₂PO₄, 2.0 g.; (NH₄)₂SO₄, 6.0 g.; glucose, 40 g.

Log-phase organisms (12 hr.) were harvested by centrifugation immediately before the experiment. They were washed twice in cold water and resuspended, either in water or in a nitrogen- and carbohydrate-free buffer (Halvorson & Spiegelman, 1952) to a density of 2.84 mg. dry wt. yeast/ml., with the aid of a Klett-Summerson colorimeter.

Inductions were carried out with organisms suspended in buffer + 3% (w/v) maltose and incubated aerobically at 30°, either in a standard Warburg apparatus or in Erlenmeyer flasks on a rotary platform shaker. α -Glucosidase activity was followed in intact organisms by the two-cup method (Halvorson & Spiegelman, 1953) or by an α -phenylglucoside assay in cell extracts (Dr S. Spiegelman, 1955, unpublished results). In the first case the amount of enzyme is proportional to the $Q_{CO_2}^0$ value corrected for the endogenous respiration. This procedure for assaying enzyme content was previously checked by a direct measurement of enzyme activity in cell extracts.

Irradiations with ultraviolet light (UV) were carried out on aqueous suspensions of organisms in open Petri dishes (5.5 in.), placed at a distance of 15 cm. from a low-pressure 15 W. General Electric germicidal bulb (2537 A.). The output of the bulb (55.3 ergs/sec. mm.²) was determined from the rate of bacteriophage inactivation (Luria & Dulbecco, 1949). After irradiations the organisms were collected, washed, and resuspended to their original volume in buffer. All manipulations were conducted under a yellow light which had previously been shown not to cause photoreactivation.

The methods described by Hershey (Hershey, 1953; Hershey, Dixon & Chase, 1953) were used for extracting the organisms and for the analysis of protein, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA). Free amino acid pools were collected and analysed by the use of specific decarboxylases (Halvorson & Spiegelman, 1952). Nucleotides of RNA were prepared (Juni, Kamen, Reiner & Spiegelman, 1948), adsorbed on Dowex 1 columns, separated by elution (Cohen, 1950), and their phosphate contents determined (Umbreit, Burris & Stauffer, 1949). The guanine and adenine of RNA nucleotides were separated by paper chromatography, eluted, and their concentrations determined from their extinction coefficients (Hershey *et al.* 1953; Dr A. D. Hershey, 1954, personal communication). Radioactivities were measured with a conventional type of end-window Geiger-Muller tube coupled to a scaling unit and a mechanical counter.

Inorganic ³²P (0.025 mg. P/mc.) was obtained from the Oak Ridge National Laboratory. Glycine-1-¹⁴C (1.21 mc./mm.) was obtained from Tracer Laboratory. Tryptozan was furnished by Dr Snyder, University of Illinois, and 2-pyridinethiol-1-oxide by Dr J. O. Lampen, Squibb Institute.

RESULTS

Dissociation by irradiation of DNA synthesis from RNA and protein synthesis

Kelner (1953) showed that dosages of ultraviolet light which abolished DNA synthesis in *Escherichia coli* had little immediate effect on RNA and protein synthesis. Similar experiments were made with yeast to determine whether this relationship held with this organism. To observe readily any differences in

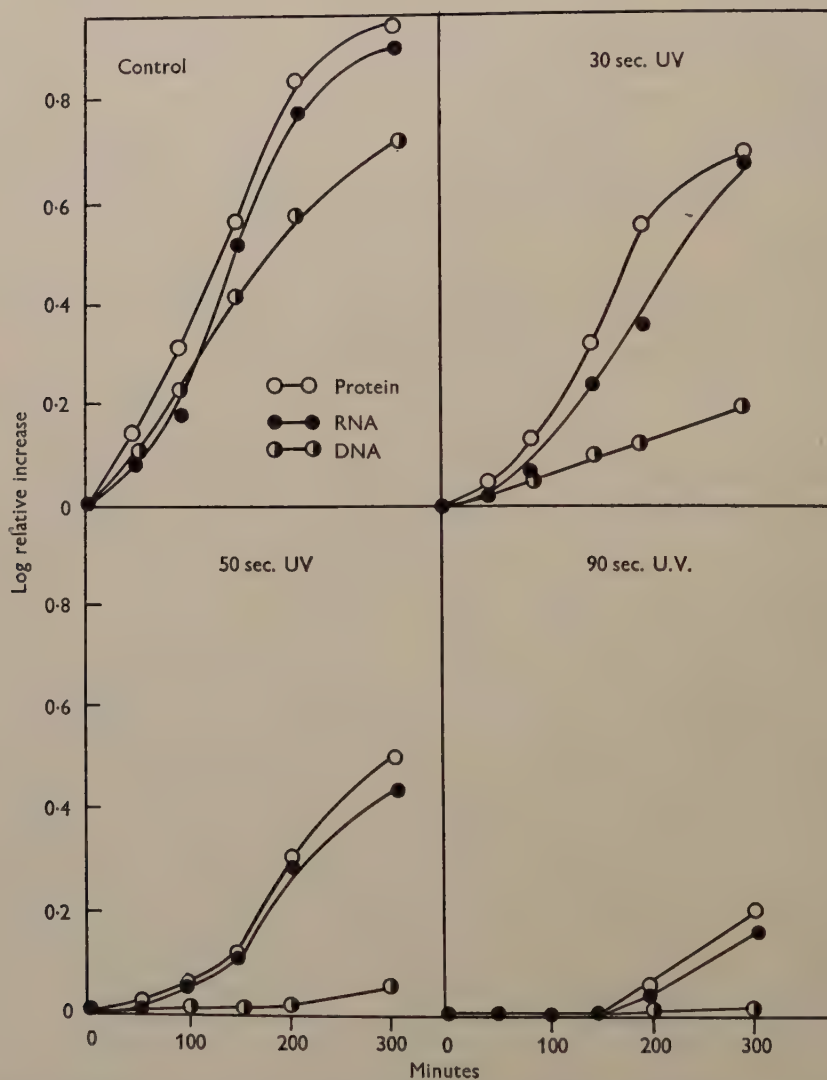


Fig. 1. Effect of UV irradiation on protein, RNA and DNA synthesis. Flasks containing 500 ml. glucose broth were each inoculated with 50 ml. of irradiated cell suspension and placed on a rotary shaker in the dark at 30°.

the UV sensitivity of protein, RNA and DNA synthesis, irradiated organisms were placed in a complete growth medium. A typical experiment was carried out as follows. Log-phase organisms were suspended in water and irradiated for various intervals. At intervals samples were removed for analysis of total protein, RNA and DNA. The results (Fig. 1) indicate a close parallel between protein, DNA and RNA synthesis in the unirradiated controls. In the organisms irradiated for 30 or 50 sec., the dissociation of DNA synthesis from protein and RNA synthesis is readily evident. Both protein and RNA synthesis are sensitive to irradiation exposures of 90 sec. or more.

Effect of irradiation on protein and enzyme synthesis in resting organisms

Induced α -glucosidase synthesis in resting organisms involves protein synthesis (Halvorson & Spiegelman, 1952), although under the conditions of induction, the total contents of RNA and DNA are constant within experimental error. One can, however, compare enzyme and protein synthesis by examining the dosages required to prevent enzyme induction and those required to inhibit net utilization of the free amino acid pool, respectively. Washed suspensions of log-phase organisms were irradiated and divided into two parts. It is clear (Fig. 2) that an excellent parallelism is observed in the loss of the capacities to incorporate amino acids into protein and to synthesize enzyme. A dosage of 80 sec. irradiation or more was sufficient virtually to abolish the synthesis of new protein. These results cannot be attributed to an inhibition of amino acid synthesis or pool replenishment from glucose and NH_3 , since dosages below 1200 sec. have little or no effect on the replenishment process (Spiegelman, Halvorson & Ben-Ishai, 1955).

It seemed desirable to examine directly the effect of irradiation on amino acid incorporation *per se* into total protein. The specific activities of the protein fractions are shown in Fig. 3, from which it is clear that a dosage of 70 sec. was sufficient to diminish glycine incorporation to a minimal value of 10%.

*Effect of ultraviolet irradiation on phosphate and glycine
incorporation into RNA*

Swenson & Giese (1950) showed that at sufficiently high dosages UV inhibited enzyme synthesis in yeast. Although the dosages required were far above those found here to be adequate for the dissociation of the effects on DNA synthesis from RNA and protein synthesis, an examination (Swenson, 1950) of the action spectrum of the inhibition of enzyme formation revealed that it coincided with the absorption spectrum of nucleic acid. Since it might be argued that the energy adsorbed by the nucleic acids is transmitted and damages some cellular processes other than RNA synthesis, it was decided to follow the effect of irradiation on the ability of RNA to incorporate ^{32}P or ^{14}C .

The effect of irradiation on the incorporation of labelled phosphate into the nucleotides of RNA was examined in an experiment, the results of which

(Table 1) indicate that rather severe effects on enzyme synthesis were achieved at dosages which had relatively little effect on the incorporation of ^{32}P into RNA. Even at the 120 sec. dose, which completely abolished enzyme synthesis and amino acid utilization, between 35 and 84% of the normal amount of

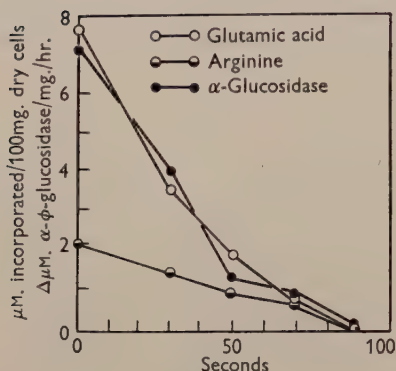


Fig. 2

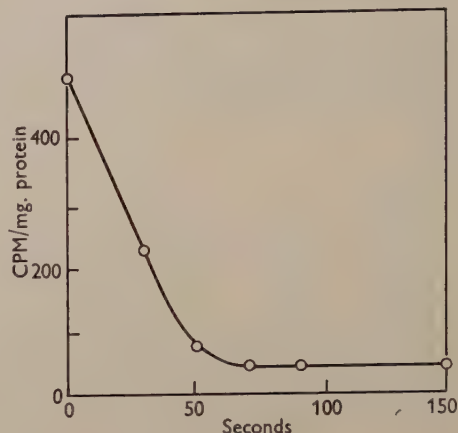


Fig. 3

Fig. 2. The effect of UV on the utilization of the free amino acid pool and α -glucosidase synthesis in yeast. The first samples were suspended in buffer containing 3% (w/v) glucose. After an 80 min. aerobic incubation at 30° , the pools were assayed for glutamic acid and arginine by the decarboxylase methods. To examine the capacity of these organisms to form enzyme, the second lot of samples were suspended in buffer containing 3% (w/v) maltose and incubated aerobically for 80 min. Following induction, dried cell preparations were made, and the α -glucosidase activity measured in the extracts by the α -phenylglucoside method.

Fig. 3. The effect of UV irradiation on the incorporation of glycine-1- ^{14}C into yeast protein. Log-phase organisms were irradiated as before and suspended to 5.68 mg. dry wt./ml. in buffer containing 3% (w/v) glucose. To 22.5 ml. samples were added 84 μg . glycine-1- ^{14}C . After aerobic incubation for 45 min. at 30° in the dark, the organisms were harvested, washed, disrupted with 0.3M-trichloroacetic acid and the protein fraction retained.

Table 1. The effect of UV irradiation on enzyme synthesis and ^{32}P incorporation into RNA

Samples (80 ml.) of cell suspensions were each irradiated for various intervals. Ten ml. of suspension were induced to form α -glucosidase for 80 min. in the presence of 3% (w/v) maltose. The remaining organisms were suspended in 70 ml. M/15 phthalate buffer (pH 4.5) containing M/450 $^{32}\text{PO}_4$ (0.35 mc.) and 3% (w/v) glucose. After aerobic incubation for 80 min. at 30° the nucleotides of RNA were collected and their specific activities and the total cell RNA content determined.

UV dose (sec.)	E^*	μg . RNA/ml.	Cytidylic		Adenylic		Uridylic		Guanylic	
			c.p.m./ μg . P	%	c.p.m./ μg . P	%	c.p.m./ μg . P	%	c.p.m./ μg . P	%
0	2.14	3.10	202	100	310	100	132	100	192	100
30	1.24	2.92	193	95	298	96	116	81	170	88
70	0.09	2.92	146	72	284	91	79	60	82	42
120	0.00	3.06	128	63	263	84	48	37	68	35

* μmole α -phenylglucoside/mg. dry wt. cells/hr.

phosphate incorporation into RNA was still observed. Within the limits of observation there was no net change in the total RNA content during the experiment.

Because the extent of incorporation is frequently a function of the labelled substrate employed (Anderson & Aquist, 1953), the effect of irradiation on the incorporation of another component of the normal metabolic pool into RNA was examined. Glycine was chosen since it is incorporated into adenine and guanine (Edmonds, Delluva & Wilson, 1952; Roberts, Abelson, Cowie, Bolton & Britten, 1955) and is readily assimilated into the free amino acid pool (Halvorson, Fry & Schwemmin, 1955). In a typical experiment (Fig. 3), irradiated organisms were incubated for 45 min. in the presence of glycine-1-¹⁴C. After isolation and hydrolysis of the RNA nucleotides, adenine and guanine were isolated and their specific activities determined. The dosages of UV which inhibited enzyme synthesis and amino acid incorporation (Fig. 3) severely depressed glycine incorporation into RNA (Table 2).

A discrepancy between phosphate (Table 1) and glycine incorporation (Fig. 2) has been observed in other systems (Anderson & Aquist, 1953). This difference may represent either the ease of phosphate exchange or a hastening of RNA breakdown by UV irradiation and a preferential utilization of RNA bases for RNA synthesis as compared to *de novo* synthesis of purines and pyrimidines. The present data do not permit a decision between these alternatives. There appears to be a fraction, variable in the case of phosphate and 11% in the case of glycine, whose incorporation is resistant to relatively high dosages of radiation. Since it was found that irradiated organisms accumulated internally both labelled phosphate and glycine, the observed effects cannot be attributed to an inhibition of cell permeability.

Table 2. *The effect of UV irradiation on glycine-1-¹⁴C incorporation into RNA*

UV dose (sec.)	Adenine		Guanine	
	c.p.m./μmole	%	c.p.m./μmole	%
0	545	100	66.5	100
30	167	30.8	35.6	53.3
50	79	14.5	14.1	21.1
70	65.6	12.1	7.5	11.2
90	65	11.9	7.0	10.4
150	67	12.3	7.2	10.7

Stages in enzyme synthesis

The probable involvement of RNA in the enzyme-forming system (Spiegelman *et al.* 1955) leads one to inquire whether its function is required throughout the cycle of enzyme synthesis. It is clear from the work of Pollock (1953) that penicillinase induction in *Bacillus cereus* involves several stages. In the first stage of primary interaction, a 60 sec. exposure to penicillin permits a subsequent linear production of enzyme, whose rate depends on the amount of bound penicillin (Pollock effect). This is followed by a 14 min. latent stage which is characterized by a UV sensitivity. The subsequent active stage of

enzyme synthesis is relatively insensitive to UV irradiation. Attempts to demonstrate clearly a Pollock effect in α -glucosidase synthesis in yeast thus far have not been encouraging, although a latent stage can be demonstrated. It is evident (fig. 4) that the UV sensitivity is related to the time of irradiation. When cells are irradiated for 80 sec. immediately before or after addition of the inducer, enzyme synthesis is completely inhibited. The same dosage of

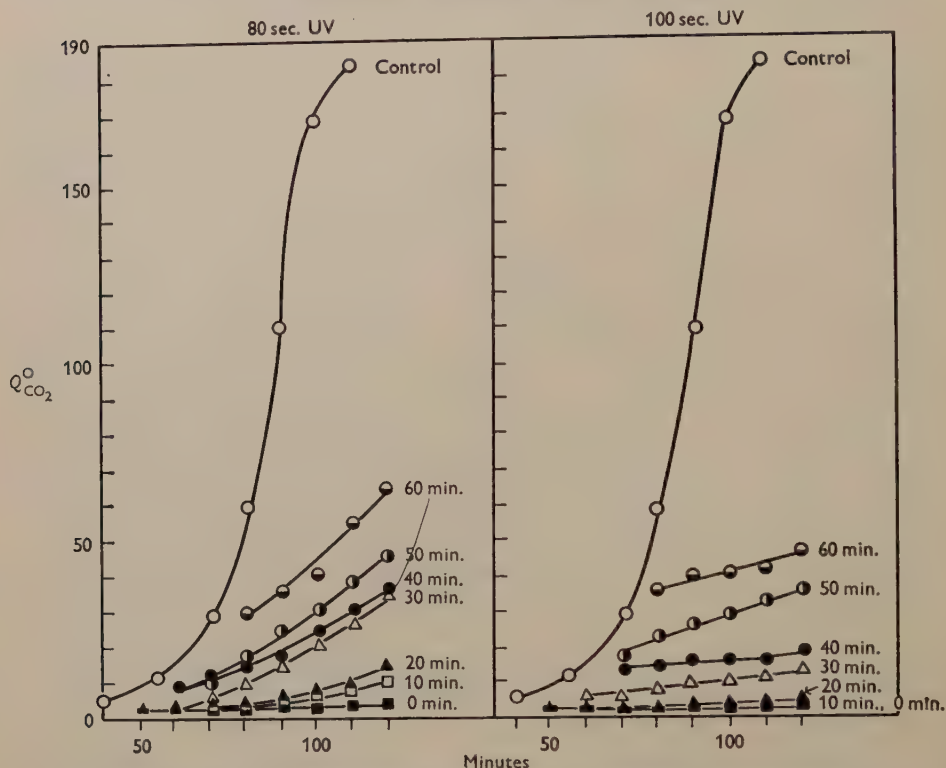


Fig. 4. Effect of the time of irradiation on the induced synthesis of α -glucosidase. UV was applied for 80 or 100 sec. at various intervals after the addition of maltose. Enzyme synthesis was followed aerobically by the two-cup method.

UV applied during the active stage (*c.* 30 min. later) only retards the rate of further synthesis. The rate of synthesis and eventual yield of enzyme are related to the time of irradiation. When dosages greater than 100 sec. are applied, this further synthesis can be prevented: under such conditions irradiation can be used to stabilize and measure the enzyme content (Spiegelman & Halvorson, 1954).

Previous investigations (Halvorson & Spiegelman, 1952; Halvorson, Spiegelman & Hinman, 1955) showed that amino acid analogues (e.g. 0.01M-*p*-fluorophenylalanine, 0.01M-tryptozan) competitively inhibited not only the net utilization of the free amino acid pool but also inhibited α -glucosidase synthesis when added simultaneously with the inducer. When 0.01M-tryptozan

was added at 20 or 40 min. after the inducer, its ability to inhibit enzyme synthesis was markedly decreased (Fig. 5). Under these conditions tryptozan still inhibited the utilization of the free amino acid pool. Similar observations were made with parallel experiments in which 0.01M-*p*-fluorophenylalanine was added at intervals after the inducer. These results present convincing evidence that the active stage is also less resistant to inhibition by amino acid analogues than is the latent stage.

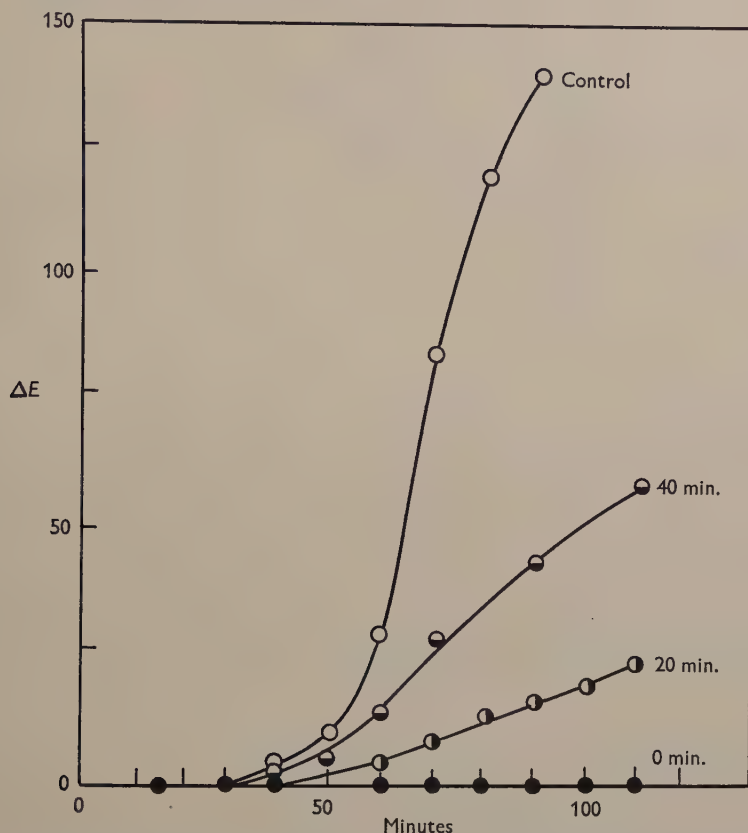


Fig. 5. Inhibition of enzyme synthesis by tryptozan. Tryptozan (final concentration of 0.01M) was added with, and 20 and 40 min. after, the addition of the inducer maltose. The increase in enzyme activity (Q_{CO_2}) over the control (ΔE) is plotted against time.

Experience over the past few years has shown that many metabolic antagonists are inactive against yeasts; fungicides, however, appear to be a promising class for analysis. Although many of these are energetic poisons, 2-pyridinethiol-1-oxide, at concentrations below 10 μ g./ml., inhibits enzyme synthesis without affecting utilization of the substrate by a fully induced yeast cell. The inhibition cannot be annulled by a mixture of vitamins, amino acids, purines and pyrimidines or an RNA hydrolysate. Although the mode of

action of this inhibitor is not understood, it is evident from the results in Table 3 that the latent stage of enzyme synthesis is more sensitive to this substance than is the active stage.

Table 3. *Effect of 2-pyridinethiol-1-oxide on the induced synthesis of α -glucosidase in yeast*

The course of enzyme production was followed by the two-cup method in the standard Warburg apparatus at 30°. 2-Pyridinethiol-1-oxide (0.5 μ g./ml.) was added at the indicated times after the addition of maltose. Enzyme activities, at the time of addition of the antagonist and at 180 min., are recorded as Q_{CO_2} .

Time of addition of 2-pyridinethiol- 1-oxide after inducer	Enzyme activity	
	At time of adding antagonist	At 180 min.
control	—	300
0	10	10
15	10	60
30	15	120
45	30	145
60	60	170

DISCUSSION

The experiments of Kelner (1953) suggested a way for deciding whether various cellular constituents are active participants of the enzyme-forming system. Kelner found in *Escherichia coli* that a virtually complete separation of RNA and protein synthesis from DNA synthesis was brought about by low dosages of UV radiation. The present investigation uses this observation to study induced α -glucosidase synthesis in yeast. Dosages of UV which inhibit DNA synthesis permit the synthesis of RNA, total protein and α -glucosidase to continue. A similar separation is probably indicated by the results of X-ray irradiation in yeast (Barron *et al.* 1953) and in tumour cells (Klein & Forssberg, 1954).

Similar separations were observed in other experiments with *Escherichia coli*. When DNA synthesis was abolished, either by the use of sulphur mustards (Sher & Mallette, 1954; Pardee, 1954), or proflavine (Drs Gros & Spiegelman, 1954; unpublished results), there was little effect on induced enzyme synthesis. Employing a thymineless mutant of *E. coli*, Cohen & Barner (1954) and Spiegelman *et al.* (1955) showed induced synthesis of xylose isomerase and β -galactosidase, respectively, in the absence of added thymine. In contrast, metabolic blocks leading to uracil and adenine deficiencies and to inhibitions in RNA synthesis resulted in the formation of little or no enzyme in the absence of the required metabolite (Pardee, 1954; Spiegelman *et al.* 1955). Experiments of this type indicate that synthesis of protein and induced enzyme is not coupled with DNA synthesis, although the presence of DNA may still be essential.

The present results, where the inhibition by varying doses of UV irradiation of total RNA synthesis (Fig. 1) and of ^{32}P (Table 1) and ^{14}C (Table 2) incor-

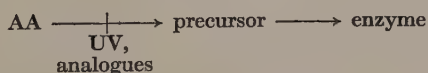
poration into RNA runs parallel with the inhibition of enzyme formation (Fig. 2) and amino acid incorporation (Fig. 3), favour the hypothesis that RNA actively participates in protein synthesis. Further evidence linking RNA and induced enzyme synthesis was provided by Spiegelman *et al.* (1955) who found that enzyme synthesis was arrested when the nucleotide pool in the wild types or in pyrimidineless or purineless mutants of yeast was depleted; the subsequent addition of purines and pyrimidines restored not only RNA but also enzyme-forming capacity. Similar results were also observed with *Escherichia coli*.

Although the synthesis of new enzyme molecules may require continued RNA synthesis, it is not clear whether the positioning of an amino acid in a protein molecule *per se* need involve RNA synthesis. The recent demonstration of a soluble protein fraction which accelerates amino acid exchange (Hoagland, 1955) stresses the necessity for distinguishing between net synthesis and exchange reactions in incorporation experiments.

In addition to the approach described above for analysing the nature of the enzyme-forming system in induction, an insight into this problem can also be gained by examining the existence and properties of the stages of enzyme synthesis in yeast. The increase in enzyme activity during induction in yeast, whether plotted on a time basis or as a Monod plot (ΔE against ΔM), is invariably preceded by a lag period (Halvorson & Spiegelman, 1955; unpublished results). This lag period is also associated with an increased sensitivity to UV irradiation, certain amino acid analogues and 2-pyridinethiol-1-oxide. Concentrations of inhibitors or dosages of UV which completely inhibit enzyme synthesis when applied at the time of induction only retard the rate when applied during active enzyme synthesis. Both the rates and yields of enzyme synthesis are related to the time of this treatment.

At least two alternative explanations may be advanced to explain the early sensitive stage of enzyme synthesis: (i) this period may involve the formation from amino acids of a precursor of the enzyme, or (ii) the synthesis of a UV-sensitive component of the enzyme-forming system, probably RNA. These two explanations need not be mutually exclusive.

In view of the specific and competitive nature of the inhibition and the parallel inhibitions of non-homologous amino acids (Halvorson & Spiegelman, 1952; Halvorson, Spiegelman & Hinman, 1955), the first alternative would require the synthesis of a precursor of the complexity of the enzyme itself. Its formation during the early stages would relieve a system depending on free amino acids and thus of sensitivity to amino acid analogues. The formation of this precursor may also be more UV-sensitive than is its conversion to enzyme:



The existence of a precursor is also supported by a comparison of the kinetics of enzyme production with the kinetics of incorporation of ^{14}C free amino acid pool components into protein (Halvorson & Spiegelman, 1955; unpublished results). A suspension of yeast cells was exposed briefly to exogenous

glycine-1- ^{14}C (during which only the free amino acid pool was labelled) washed and induced to form α -glucosidase in the absence of exogenous nitrogen. After 40 min. of induction the ^{14}C incorporation into protein had reached its maximal value while α -glucosidase induction was only one-third complete. These results suggest that the nitrogen for the remaining enzyme, which appeared after 40 min., was derived from the free amino acid pool during the first 40 min.

Until such a precursor can be isolated its existence must be considered as a speculation. The induction of β -galactosidase in *Escherichia coli* has been the only system thus far examined for the existence of a non-enzymic protein precursor. Although a non-enzymic protein fraction was detected which was immunologically related to the enzyme, it has been shown not to be a precursor of the enzyme (Rotman & Spiegelman, 1954; Hogness, Cohn & Monod, 1955).

An alternative possibility to explain the early sensitive stage in induction might be the existence of an essential UV-sensitive component of the enzyme-forming system. This might be assumed to be either RNA itself or an RNA-forming component of the enzyme-forming system. If RNA and protein synthesis are mutually dependent, then the amino acid analogues should also inhibit RNA synthesis and their effects would not necessarily favour the hypothesis of a precursor. The observation by Gale & Folkes (1953) that in bacteria chloramphenicol can inhibit protein but not nucleic acid synthesis does not support this view. However, it is not clear at the present time whether RNA synthesis in yeast is normally dependent upon protein synthesis. The present data also do not exclude the possibility that the sensitive stage involves both the formation of a precursor and the synthesis of a necessary component of the enzyme-forming system.

We wish to express our deep appreciation to Dr J. O. Lampen (Squibb Institute) for the supply of 2-pyridinethiol-1-oxide and Mr Manuel Rosenbaum for technical assistance in these studies. This investigation was aided by a grant from United States Public Health Service and a grant by the Phoenix Project at the University of Michigan. One of us (L.J.) held a United States Public Health Fellowship during the course of the work.

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A Study of the Genetics of Penicillin-producing Capacity in *Penicillium chrysogenum*

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SUMMARY: Four non-penicillin-producing (*p*) strains were isolated from the strain 47.1564 Wis. of *Penicillium chrysogenum* and used in this work. Seven heterokaryons and seven heterozygous diploids were synthesized between the four *p* strains and one penicillin-producing (+) strain. All these strains were further marked by nutritional requirements and differences in colour of the conidia. None of the heterokaryons and diploids between non-producing strains produced any appreciable amount of penicillin; all the heterokaryons and diploids between a producing and a non-producing strain did produce penicillin in amounts of the same order as those of the producing strain. The four *p* mutations behave like alleles: *p* is recessive to the wild condition. Linkage of the allele *p* to *y* and to *cy* may be deduced from data of the somatic segregation from a heterozygous diploid.

Bonner (1947) was unable to restore penicillin production in non-penicillin-producing mutants by growing numerous pairs of such strains in mixed cultures. Using parasexual recombination in *Penicillium chrysogenum* (Pontecorvo & Sermonti, 1953, 1954; Sermonti, 1954*a, b*) it is now possible to analyse heterokaryotic, heterozygotic and possibly allele interactions between mutants differing in penicillin production. The present paper reports an investigation to this end.

METHODS

Unless otherwise stated, the techniques, media, etc., used in the present work are the routine ones for genetics and fermentation researches in *Penicillium chrysogenum*, as published in detail elsewhere (Sermonti, 1954*a, b*; Camici, Sermonti & Chain, 1952). Penicillin production on agar was obtained with the basal medium of Jarvis & Johnson (1950) or the 'E' medium of Raper, Dorothy & Coghill (1944). Small plugs of agar were removed close to the colony to be tested and placed on agar inoculated with a test organism (Raper *et al.* 1944). Only presence or absence of the inhibition halo was taken into account. Penicillin production in submerged culture was tested by a modification of the cylinder-plate method of Schmidt & Moyer (1944). In both tests the I.C.I. strain of *Bacillus subtilis* was used.

Mutant production. All the mutants used in the course of the work were derived from strain 47.1564 Wis. of *Penicillium chrysogenum*, which will be referred to as the wild type. Mutants were isolated after ultraviolet (UV) irradiation of the conidia in a dosage which gave 95% mortality.

Colour mutants were identified visually among the colonies which grew from the surviving conidia. Nutritional mutants (auxotrophs) were identified

by the total isolation method (Fries, 1948). Non-penicillin-producing strains were identified by transferring single colonies grown from irradiated conidia to small Petri dishes and then testing the cultures for penicillin production.

Table 1 gives a list of the mutant strains used, with their characteristics.

Table 1. *Characteristics and origins of the mutants employed in the course of the research*

Strain symbol*	Parent strain	Mutagenic agent
97 p_1	47.1564 Wis.	Ultraviolet
99 p_2	47.1564 Wis.	Ultraviolet
15 y_1	47.1564 Wis.	Ultraviolet
20 w	47.1564 Wis.	Mustard gas†
123 $y_2 p_1$	97 p_1	Ultraviolet
42 $y_3 p_2$	99 p_2	Ultraviolet
92 $p_3 y_1$	15 y_1	Ultraviolet
46 $me w$	20 w	Ultraviolet
124 $hy y_2 p_1$	123 $y_2 p_1$	Ultraviolet
122 $hy y_3 p_2$	42 $y_3 p_2$	Ultraviolet
65 $pr y_3 p_2$	42 $y_3 p_2$	Ultraviolet
63 $cy p_3 y_1$	92 $p_3 y_1$	Ultraviolet
121 $p_4 me w$	46 $me w$	Ultraviolet

* The strain symbols consist of a code number followed by symbols whose interpretation is as follows: p = absence of penicillin production; y = yellow colour of conidia; me = methionine requirement; hy = hypoxanthine or adenine requirement; pr = proline requirement; cy = cysteine or methionine requirement. Mimic mutants of similar phenotype representing different mutations are distinguished by a subscript.

† According to the technique of Stahmann & Stauffer (1947).

Auxotrophic mutants appeared at an average rate of rather less than 1% of the survivors; mutants not producing penicillin (p) at a rate of about 0.25%.

The four p mutants studied were chosen from a total of ten isolated after testing 3962 colonies which had survived UV irradiation. This kind of mutant was obtained by Bonner (1947) at the rate of 0.2% after UV irradiation and at the rate of about 0.5% by Roegner, Stahmann & Stauffer (1954) by the use of methyl-*bis*-(β -chloroethyl)amine.

All the non-penicillin-producing strains used in this work were checked for non-production in submerged culture. Strain 121 p_4 produced about half a penicillin unit/ml.; strains 97 p_1 and 99 p_2 produced smaller quantities which cannot be measured with precision, and strain 92 $p_3 y_1$ produced no penicillin at all, compared with 250 u./ml. for the wild type control. No appreciably higher penicillin yields were obtained in later tests, even when better yields were obtained from the control.

RESULTS

Synthesis of balanced heterokaryons

Nine balanced heterokaryons (Table 2) were obtained with the last six marked strains listed in Table 1. The combinations were devised to combine the p markers two by two in all possible ways, and two further combinations

were those of a normal penicillin-producing strain with each of two strains with different *p* markers.

Table 2. *Balanced heterokaryons and the media used to obtain them**

Balanced heterokaryon	Medium†
63 <i>cy p</i> ₃ <i>y</i> ₁ + 46 <i>me w</i>	MM + methionine
63 <i>cy p</i> ₃ <i>y</i> ₁ + 65 <i>pr y</i> ₃ <i>p</i> ₃	MM + methionine + proline
63 <i>cy p</i> ₃ <i>y</i> ₁ + 122 <i>hy y</i> ₃ <i>p</i> ₂	A corn steep medium‡
63 <i>cy p</i> ₃ <i>y</i> ₁ + 121 <i>p</i> ₄ <i>me w</i>	MM + methionine
63 <i>cy p</i> ₃ <i>y</i> ₁ + 124 <i>hy y</i> ₃ <i>p</i> ₁	MM + methionine + adenine
124 <i>hy y</i> ₃ <i>p</i> ₁ + 65 <i>pr y</i> ₃ <i>p</i> ₃	MM + adenine + proline
124 <i>hy y</i> ₃ <i>p</i> ₁ + 121 <i>p</i> ₄ <i>me w</i>	MM + adenine + methionine
65 <i>pr y</i> ₃ <i>p</i> ₃ + 121 <i>p</i> ₄ <i>me w</i>	MM + proline + methionine
65 <i>pr y</i> ₃ <i>p</i> ₃ + 46 <i>me w</i>	MM + proline + methionine

* Balanced heterokaryons were selected as tufts of better growing mycelium in the zone of contact between two colonies of different types.

† Additions to minimal medium (MM) for growth of mutants were made at the following concentrations (mg./l.): DL-methionine, 10; L-proline, 10; adenine, 1.25.

‡ MM supplemented by 1% (w/v) corn steep liquor.

Heterokaryons were synthesized by the technique developed by Sermonti (1954a) for *Penicillium chrysogenum*. The nine clones synthesized in this way were shown to be heterokaryons by their ability to give rise to heterozygous diploids. This was further confirmed for some of them by the reappearance of the parent types after plating their conidia on nutritionally complete medium (CM) (63 + 121; 65 + 121 and 46 + 63); by absence of growth from conidia and occurrence of growth from mycelium transfers on minimal medium (MM) (63 + 65; 63 + 122; 63 + 121; 63 + 124; 65 + 121 and 46 + 65); and by recovery of the parent types from conidia of colonies derived after the isolation of single hyphae (46 + 63).

Selection of heterozygous diploids

A list of heterozygous diploids is given in Table 3; each diploid was obtained from one of the heterokaryons listed in Table 2, either after plating conidia on MM medium or as sectors from heterokaryon colonies (Roper, 1952). All the heterozygous diploid strains were purified by single conidium isolation with a micromanipulator.

The ability of the strains isolated to give rise to segregants carrying the recessive characters of the parent strains, either combined or in isolation (see Table 7), constitutes a definite demonstration of their being heterozygous diploids.

Characteristics of heterokaryons and of heterozygous diploids

The general properties of heterokaryons and heterozygous diploids have been the subject of investigations reported in previous papers. Colour of the sporng surface and presence or absence of penicillin production alone will here be described in detail.

Colour. The colour of the sporng colony surface in the case of heterokaryons is intermediate between those of the parent strains (see Table 4). The present

observations confirm those already reported by Pontecorvo & Sermoniti (1954) and by Sermoniti (1954*a*). The sporling surfaces of heterozygous diploids are coloured various shades of green, i.e. similar to the colour of the wild type: the diploid 63 *cy p₃ y₁/124 hy y₂ p₁* is an exception to this rule in having yellow

Table 3. *Heterozygous diploids obtained from balanced heterokaryons*(a) *Diploids obtained by plating conidia of heterokaryons on minimal medium*

Heterozygous diploids*	No. of conidia of the heterokaryon plated		No. of diploids obtained	Ratio of conidia plated: diploids obtained
	Total	Per dish		
VI 63 <i>cy p₃ y₁/65 pr y₃ p₂</i>	18.8×10^6	13.5×10^5	1400	13,400:1
IX 63 <i>cy p₃ y₁/122 hy y₃ p₂</i>	9.8×10^6	6.1×10^5	5000	1,960:1
XIII 63 <i>cy p₃ y₁/121 p₄ me w</i>	0.36×10^6	0.3×10^5	6	60,000:1
XV 63 <i>cy p₃ y₁/124 hy y₂ p₁</i>	1.5×10^6	2×10^5	622	2,410:1

(b) *Diploids obtained as green sectors from balanced heterokaryons*

VI 63 *cy p₃ y₁/65 pr y₃ p₂*
 VII 63 *cy p₃ y₁/46 me w*
 VIII 124 *hy y₂ p₁/65 pr y₃ p₂*
 IX 63 *cy p₃ y₁/122 hy y₃ p₂*
 X 65 *pr y₃ p₂/121 p₄ me w*
 XI 124 *hy y₂ p₁/121 p₄ me w*
 XII 65 *pr y₃ p₂/46 me w*

* Diploids are symbolized by the symbol of the heterokaryon from which they are derived, but with the + replaced by /. The Roman numerals in front of the symbol give the code numbers of the diploids.

Table 4. *Colour of sporling surface in parent strain, heterokaryons and heterozygous diploids*

Parent strains*		Colour of conidia in parent strains		Colour of sporling surface of <i>a+b</i> heterokaryons	Colour of conidia of <i>a/b</i> diploids
<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>		
63 <i>y₁</i>	46 <i>w</i>	Yellow†	White	Yellowish white	Grey-green‡
63 <i>y₁</i>	65 <i>y₃</i>	Yellow	Yellow	Yellow	Yellowish green
63 <i>y₁</i>	122 <i>y₃</i>	Yellow	Yellow	Yellow	Yellowish green
63 <i>y₁</i>	121 <i>w</i>	Yellow	White	Yellowish white	Grey-green
63 <i>y₁</i>	124 <i>y₂</i>	Yellow	Yellow	Yellow	Yellow
124 <i>y₂</i>	65 <i>y₃</i>	Yellow	Yellow	Yellow	Yellowish green
124 <i>y₂</i>	121 <i>w</i>	Yellow	White	Yellowish white	Grey-green
65 <i>y₃</i>	121 <i>w</i>	Yellow	White	Very pale yellow	Grey-green
65 <i>y₃</i>	46 <i>w</i>	Yellow	White	Off-white	Grey-green

* Symbols not concerned with colour are omitted.

† All the yellow strains assume a tinge of pink after a time.

‡ All the green strains tend to turn brown after a time.

conidia. Diploids between two yellow strains are yellowish-green in colour, except in the case of 63/124; five diploids established between a yellow and a white strain were of a grey-green colour slightly paler than the wild type (see Table 4).

Penicillin production. Qualitative data relative to penicillin production on agar by heterokaryons and by heterozygous diploids agree in every case. With the strains used, heterokaryons and diploids between a producing strain and a non-producing strain produced penicillin; heterokaryons and diploids between two non-producing strains did not produce appreciable amounts of penicillin.

Table 5 gives quantitative results for penicillin production in submerged culture of heterozygous diploids as compared with the wild type (47.1564 Wis.) and with a diploid homozygous for penicillin production obtained from two strains derived from the wild type (26 *le*/29 *pr*).

Table 5. *Penicillin production in submerged culture by heterozygous diploids compared with that by strain 47.1564 Wis. (haploid)*

Average yields of penicillin in three flasks shaken on rotary shaker are given. The standard errors are given as well as the values. Medium: corn steep + lactose. Precursor added every 12 hr. from the 24 hr.

Strain	Genotype*	Penicillin production (units/ml. at 96 hr.)
47.1564 Wis.	+	441 ± 14
III 26/29†	+ / +	361 ± 8
XII 46/65	+ / p_2	358 ± 16
IV 51/63‡	+ / p_3	238 ± 9
VII 46/63	+ / p_3	236 ± 5
VIII 124/65	p_1 / p_2	Traces
XV 63/124	p_3 / p_1	0
XI 121/124	p_4 / p_1	Traces
X 65/121	p_2 / p_4	Traces
VI 63/65	p_3 / p_2	0
IX 63/122	p_3 / p_2	0
XIII 63/121	p_3 / p_4	0

* Only symbols concerning the gene *p* / + are given.

† Parent strains: 26 *le* (leucine) and 29 *pr*, both derived from 47.1564 Wis.

‡ Parent strains: 63 *cy p₃ y₁* and 51 *pr thi* (thiamine), which also derives from 47.1564 Wis.

The results of submerged culture tests give substantial confirmation of the agar tests. Some *p/p* diploids produce traces of penicillin, but the maximum is four units compared with the 414 units of the wild type control. The two +/*p* heterozygotes produce considerable quantities of penicillin. The diploid III +/+ produces about the same as the haploid wild type.

Segregants from heterozygous diploids

Segregants for colour or nutritional requirements have been isolated (Sermonti, 1954b) from all the strains described as heterozygous diploids in the present paper. This definitely confirms their heterozygosis.

Table 7 gives the results of a detailed segregation analysis carried out on the diploids VII 63 *cy p₃ y₁*/46 *me w* and XIII 63 *cy p₃ y₁*/121 *p₄ me w*. The only difference between the two diploids is that XIII contains the allele *p₄*, which

distinguishes strain 121 from its parent strain 46. The segregants were selected for colour of conidia according to the method already described (Sermonti, 1954*b*).

In both experiments it was found that the yellow segregants either were prototrophic or had a cysteine requirement, while the white segregants either were prototrophic or had a methionine requirement. All the yellow segregants from diploid VII were non-producing and all the white segregants produced penicillin. Neither white nor yellow segregants from diploid XIII produced penicillin.

Extensive experiments were carried out in an attempt to select a penicillin-producing segregant from a non-producing diploid. Diploid X *65 pr y₃ p₂/121 p₄ me w* was tried first. Out of about 6000 colonies subjected to a preliminary test, and forty-two further checked, not a single penicillin-producing segregant was obtained.

The work was continued using the diploid XIII *63 cy p₃ y₁/121 p₄ me w*. Out of 14,174 colonies tested a single penicillin-producing colony (308.6906) was detected. The penicillin production of this strain was tested in submerged culture (Table 6) and gives results similar to those of diploid VII.

Table 6. *Submerged culture penicillin production by strain 308.6906 compared with production by diploid VII and strain 47.1564 Wis.*

Average yields of penicillin in three flasks shaken on rotary shaker are given. The standard errors are given as well as the values. Medium: corn steep-lactose. Precursor added every 12 hr. from the 24 hr.

Strain	Yield of penicillin (units/ml.)	
	At 96 hr.	At 120 hr.
308.6906	340 ± 17	369 ± 2
VII 46/63	297 ± 10	249 ± 11
47.1564 Wis.	489 ± 11	556 ± 20

The isolated strain proved to be green and prototrophic. It was genetically analysed by an examination of its second-order segregants selected for colour. Table 7 gives the details of the experiment.

It appears from Table 7 that, unlike the segregants of diploid VII, all the yellow segregants of this strain, and none of its white segregants, produced penicillin.

Some of the white segregants are prototrophic and some require methionine, but no yellow segregant was found with a cysteine requirement. The cysteine requirement was also looked for in segregants selected for nutritional requirements (Sermonti, 1954*b*). 426 out of the 1440 colonies examined were tested for nutritional requirements: 73 showed methionine requirement, but not one showed a cysteine requirement. Two yellow prototrophic segregants originating from strain 308.6906 were investigated for further segregation, but not one colony with a cysteine requirement appeared after testing 455 colonies selected from 3252.

Table 7. *Segregants from heterozygous diploids VII and XIII and from strain 308.6906 of Penicillium chrysogenum.*

The segregants were selected for colour, purified and then tested for nutritional requirements and penicillin production.

		Penicillin production	
Colour	Nutritional requirement	Present (no.)	Absent (no.)
A. First-order segregants* from diploid VII 63 <i>cy p₃ y₁/46 me w</i>			
Yellow	Cysteine	—	4
	None	—	17
White	Methionine	20	—
	None	19	—
B. First-order segregants† from diploid XIII 63 <i>cy p₃ y₁/121 p₄ me w</i>			
Yellow	Cysteine	—	13
	None	—	3
White	Methionine	—	22
	None	—	2
C. Second-order segregants from strain 308.6906			
Yellow‡	None	10	—
White§	Methionine	—	16
	None	—	4

* Selected from a total of 10,185 colonies examined.

† Selected from a total of 15,130 colonies examined.

‡ Selected from a total of 9,630 colonies examined.

§ Selected from a total of 951 colonies examined.

DISCUSSION

The genetic determination of the difference between presence and absence of capacity to produce penicillin is clear from the results given above, and in particular from the data on the segregation of diploid VII and of strain 308.6906. Absence of penicillin production (*p*) segregates from penicillin-producing heterozygous diploids as determined by a single difference in a chromosome region. Absence is recessive to presence of penicillin production. Quantitative tests in submerged culture of the penicillin production of strains segregating from these diploids have not been carried out, but the evidence of the qualitative tests on agar cultures is sufficient indication of this segregation. Of the colour-selected segregants some showed no trace of production, while others showed rates of production similar to those of the parent type.

Linkage of the character *p* with other characters may be deduced from the segregation data of diploid VII. In terms of mitotic segregation (Pontecorvo, Tarr Gloor & Forbes, 1954) these data would mean that *p* is distal to *y*, which would be in turn distal to *cy*.

The proportion of *p* mutants—0.25% in the present work, 0.20% in Bonner's (1947) work—is very high, considerably higher than the proportion of any mutant for a specific nutritional requirement obtained in *Penicillium chrysogenum*. At least three of the four *p* alleles (*p*₁ or *p*₂, *p*₃ and *p*₄) are

certainly the result of independent mutation, since they were obtained in differently marked strains (see Table 1). p_1 , p_3 and p_4 determine phenotypes which are slightly different (though p_1 and p_2 are phenotypically indistinguishable). Mutant p_3 , which determines complete absence in the diploids p_3/p , also determines complete absence of penicillin in the haploid and reduced production in the $p_3/+$ diploids (see Table 5).

In diploids synthesized between pairs of non-producing strains the four p behave as if they were allelic: i.e. practically no penicillin is produced by any of the six combinations between different non-penicillin-producing strains. Similarly, two mutations for yellow colour of conidia, y_1 and y_2 , behave as if they were allelic. The green colour of the conidia does not reappear in diploid XV, which is heterozygous, y_1/y_2 . However, the third yellow mutant, y_3 , is not allelic to the two just mentioned because the heterozygous diploids VI y_1/y_3 and XI y_2/y_3 have green conidia like the wild type. Whether all the p mutants and two of the y mutants, y_1 and y_2 , should be called alleles or pseudo-alleles—considering, amongst other things, the possible origin of 308.6906 by crossing-over—is a matter of definition, and is not worth while discussing at the present stage of genetic analysis of *Penicillium chrysogenum*. On whatever interpretation, all the mutants studied which block penicillin synthesis concern a single gene. Two such genes, inactivated by different mutations, could not restore penicillin production when together in a heterozygote or in a heterokaryon.

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The Influence of Depletion of Nitrogenous Reserves upon the Phenomenon of Induced Enzyme Biosynthesis in Cells of *Escherichia coli*

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SUMMARY: No β -galactosidase was formed when nitrogen-depleted cells of *Escherichia coli* 1433 were treated with lactose unless a source of nitrogen was added to the cells. In the presence of an exogenous nitrogen source there was an appreciable delay between the addition of lactose and the appearance of induced β -galactosidase activity. In contrast, induced increases of nitrate- and tetrathionate-reductase activities were developed without appreciable delay when depleted cells were treated with nitrate and tetrathionate, respectively, in the absence of additional nitrogen sources. The total amounts of the reductase activities developed in depleted cells were less than those obtained with non-depleted cells. The total amount of nitrate reductase activity formed was only slightly increased in the presence of ammonium sulphate, whereas normal degrees of activity were attained in the presence of casein hydrolysate. Ammonium sulphate markedly stimulated the formation of tetrathionate reductase activity.

Conclusive evidence has been presented (Monod & Cohn, 1953; Hogness, Cohn & Monod, 1955; Rotman & Spiegelman, 1954) that under certain conditions cells of *Escherichia coli* do not contain appreciable amounts of complex precursors of the inducible (adaptive) β -galactosidase. Similarly, for the inducible maltozymase system of *Saccharomyces cerevisiae*, Halvorson & Spiegelman (1952, 1953*a, b*; Spiegelman & Halvorson, 1953) concluded that induced enzyme is probably not formed via complex precursors that are normally present in non-induced cells. It has, therefore, been suggested (Monod & Cohn, 1953; Rotman & Spiegelman, 1954) that the process of induced enzyme formation is essentially one of *de novo* synthesis entirely from free amino acids. However, the maltozymase system of *Saccharomyces cerevisiae* is unstable and can be degraded even by cells which are not depleted of nitrogen reserves (Spiegelman & Dunn, 1947). Hence, as noted by Spiegelman & Halvorson (1953), the information obtained to date for this system cannot be considered as critical evidence of the absence of complex enzyme precursors from the cells.

The processes of induced β -galactosidase synthesis in *Escherichia coli* differ in a number of respects from those of induced formation of other systems. For example, little β -galactosidase synthesis can occur in suspensions of well-washed cells of *E. coli* (Cohn & Torriani, 1953), whereas considerable formation of the nitrate and tetrathionate reductase (nitratase and tetrathionase)

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systems can occur in such cells (Pollock, 1946). We have, therefore, compared the effects of depletion of the nitrogen reserves upon the formation of the inducible β -galactosidase and of nitrate and tetrathionate reductases by cells of *E. coli* in order to determine whether the processes of formation of the various enzymes also differ under conditions of acute shortage of available nitrogen. Marked differences were found between the processes of synthesis of β -galactosidase and those of formation of the reductases.

METHODS

Organism and medium. All experiments were performed with the 1433 strain of *Escherichia coli* (Pollock, 1946).

The medium used was based upon the medium 56 of Monod, Cohen-Bazire & Cohn (1951), but with the N-content decreased to a growth-limiting concentration of 0.02% (w/v) of $(\text{NH}_4)_2\text{SO}_4$. It consisted of: KH_2PO_4 , 13.6 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g.; CaCl_2 , 0.01 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0005 g.; KOH to pH 7.2 and water to 1 l. Double-distilled water was used throughout.

Preparation of cultures for induction experiments. Batches of medium were supplemented with 0.05% (w/v) nitrogen-free maltose, inoculated and incubated aerobically at 37° overnight. Additional maltose was added to a concentration of 0.5% (w/v) and the culture re-incubated aerobically at 37°. Cultures were used for induction experiments 3 hr. after growth had ceased because of exhaustion of the nitrogen source.

Growth measurement. Growth was followed by measurements of optical density at a wavelength of 610 μ . and the readings converted to mg. dry wt. of cell mass by means of standard curves.

β -Galactosidase adaptation. Aerobic formation of enzyme was induced in 125 ml. Erlenmeyer flasks shaken at 37°. Each flask contained 6 or 7 ml. of depleted culture, recrystallized lactose (Pfanstiehl C.P.) to 0.2% (w/v), additions and distilled water to 10 ml. After incubation for the desired period, the optical density was measured and the culture prepared for assay of enzyme activity by incubating with 2 drops of toluene at 37° for 15 min.

Anaerobic formation of the enzyme was similarly induced in evacuated Thunberg tubes, the contents being transferred to Erlenmeyer flasks for treatment with toluene.

β -Galactosidase activities were determined, at room temperature (*c.* 25°), by the liberation of *o*-nitrophenol from *m*/600 *o*-nitrophenyl- β -D-galactoside (Lederberg, 1950). Activities are expressed as μ mole galactoside hydrolysed/hr./mg. dry wt. cells. Total activities of the cultures are expressed as μ mole galactoside hydrolysed/hr./10 ml. culture.

Nitrate reductase adaptation. Nitrate reductase formation was induced in evacuated Thunberg tubes at 37°. Unless otherwise stated, each tube contained 6 or 7 ml. depleted culture, 1 ml. 0.1M-glucose (twice recrystallized from 80% (v/v) ethanol), 0.5 or 1 ml. of 0.1M-sodium nitrate in the stopper, additions and distilled water to 10 ml. The adaptation was followed by estimations of the nitrite produced using the Griess-Ilosvay reagent (Pollock, 1946).

The cells were spun down, washed once with distilled water and resuspended in water.

Nitrate reductase activities were determined in evacuated Thunberg tubes at 37° with 0.01M-sodium formate as H-donor and in the presence of M/800 FeSO₄ (Wainwright & Pollock, 1949). Activities are expressed as μ mole nitrite produced/hr./mg. dry wt. cells. Total nitrate reductase activities of the cultures are expressed as μ mole nitrite produced/hr./10 ml. culture.

Tetrathionate reductase adaptation. Tetrathionate reductase formation was induced in evacuated Thunberg tubes in the same manner as nitrate reductase, 0.1M Na tetrathionate replacing the nitrate.

Tetrathionate reductase activities were determined in evacuated Thunberg tubes at 37° by titration of the thiosulphate produced with 0.002N iodine (Wainwright & Pollock, 1949). Activities are expressed as μ mole thiosulphate produced/hr./mg. dry wt. cells. Total tetrathionate reductase activities of the cultures are expressed as μ mole of thiosulphate produced/hr./10 ml. culture.

RESULTS

β -Galactosidase adaptation as a control of experiments

Cohn & Torriani (1953) found that organisms of the ML 30 strain of *Escherichia coli* which had been depleted of nitrogenous reserves did not form any β -galactosidase when treated with inducer. Similarly, we found that nitrogen-depleted organisms of the 1433 strain did not form any β -galactosidase when treated with lactose for periods up to 4 hr. under either aerobic or anaerobic conditions. Indeed, the enzyme activity frequently fell below the small 'basal' activity originally present. In the presence of 0.5% (w/v) (NH₄)₂SO₄ enzyme formation did occur after a lag period, which was invariably shorter under anaerobic conditions than under aerobic conditions (Fig. 1); the experiment illustrated records the shortest lag we have observed aerobically and in one experiment the lag exceeded 3 hr.

Table 1. *Anaerobic formation of β -galactosidase in the presence of a low concentration of ammonium sulphate*

Cultures depleted of nitrogen reserves were incubated for 2.5 hr. anaerobically at 37° with 0.2% (w/v) lactose in the absence or presence of 0.0005% (w/v) (NH₄)₂SO₄.

β -galactosidase activities (μ mole/hr./mg. dry wt.)

Initial culture	Without (NH ₄) ₂ SO ₄	With (NH ₄) ₂ SO ₄
0.10	0.05	1.82
0.22	0.24	3.60
0.16	0.08	0.18
0.17	0.18	0.43
0.17	0.07	0.68

Marked increases in β -galactosidase activity were observed after anaerobic induction in the presence of concentrations of (NH₄)₂SO₄ as low as 0.0005% (w/v) (equivalent to 1.1 μ g. N/ml.) (Table 1) and occasionally, but not invariably,

with concentrations of 0.00005% (w/v). However, we never observed any increase in β -galactosidase activity after aerobic induction for 3 hr. in the presence of concentrations of 0.0005% (w/v) $(\text{NH}_4)_2\text{SO}_4$ or less.

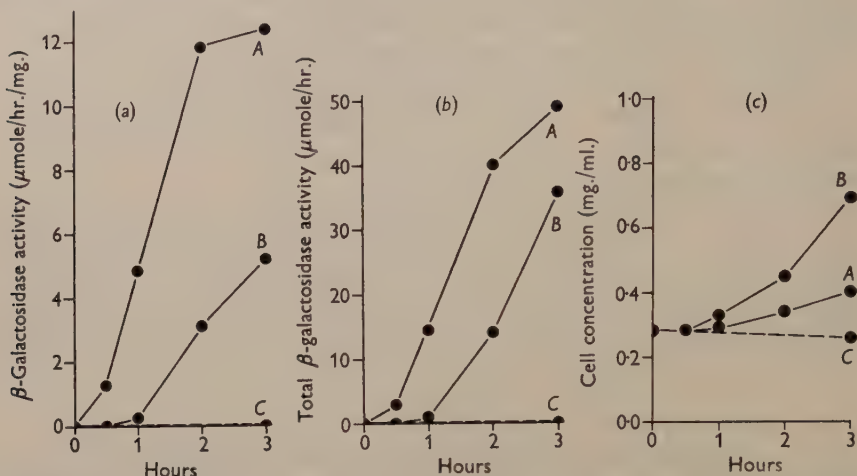


Fig. 1. Influence of $(\text{NH}_4)_2\text{SO}_4$ (0.5% w/v) on aerobic and anaerobic formation of β -galactosidase by nitrogen depleted *Escherichia coli* 1433 induced with lactose (0.2% w/v). (a) β -galactosidase activities/mg. dry wt. cells; (b) total β -galactosidase activities of cultures; (c) growth curves. A, anaerobic induction; B, aerobic induction; C, anaerobic induction without $(\text{NH}_4)_2\text{SO}_4$.

For routine control of the depletion of the nitrogen reserves, in every experiment reported here we incubated portions of the cultures aerobically with 0.2% (w/v) lactose for a minimum period of 2 hr. and confirmed the absence of β -galactosidase formation. In many of the experiments we also incubated portions of the culture anaerobically with lactose as a control to confirm the absence of detectable traces of contaminating nitrogen sources.

Nitrate reductase formation in nitrogen-depleted organisms

When depleted organisms were incubated anaerobically with nitrate and glucose (as energy source) there was a rapid and marked increase in nitrate reductase activity, and the total activity of the culture rose to a maximum value within 2 hr. Fig. 2 shows the results of an experiment carried out with the same batch of depleted cells used for the experiment illustrated in Fig. 1. There was some growth during the adaptation (Fig. 2c), indicating that the cells had been supplied with a source of available nitrogen, which was identified as being produced metabolically from the inducing nitrate. As the inducing nitrate was itself serving as a source of available nitrogen, it was clearly not possible to study nitrate reductase formation in the total absence of nitrogen sources. However, it was consistently found that there was little or no lag in formation of nitrate reductase, whereas there was an appreciable delay in the onset of increase in cell mass (Fig. 2a, c). Further, growth continued at an

undiminished rate after the maximum total nitrate reductase activity of the culture was attained, indicating that the latter was not limited by exhaustion of the source of available nitrogen. Indeed, there was little increase in the maximum total activity of the culture when $(\text{NH}_4)_2\text{SO}_4$ was added in concentrations of 0.005 (Fig. 2*b*), 0.05 and 0.5% (w/v), although the initial rate of increase in enzyme activity was enhanced in every case (Fig. 2*a, b*).

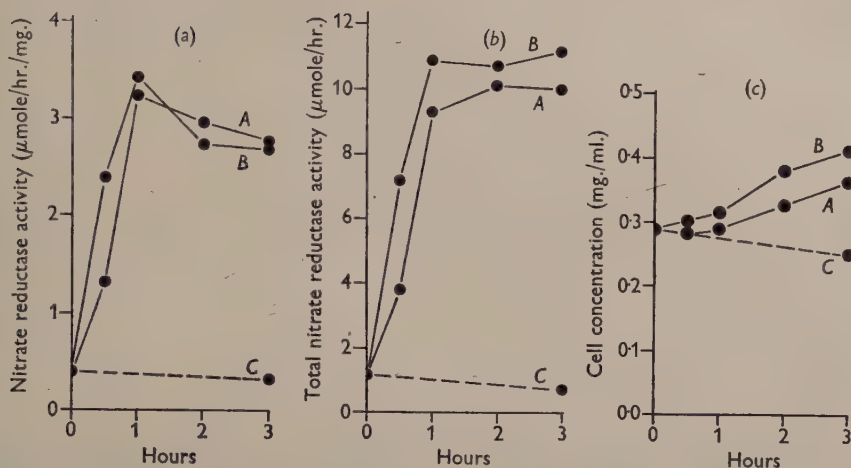


Fig. 2. Influence of $(\text{NH}_4)_2\text{SO}_4$ (0.005%, w/v) on nitrate reductase formation by nitrogen depleted *Escherichia coli* 1433 induced with nitrate (0.01M). (a) Nitrate reductase activities per mg. dry wt. of cells; (b) total nitrate reductase activities of cultures; (c) growth curves. A, without $(\text{NH}_4)_2\text{SO}_4$; B, with $(\text{NH}_4)_2\text{SO}_4$; C, control without nitrate or $(\text{NH}_4)_2\text{SO}_4$.

Even in the presence of $(\text{NH}_4)_2\text{SO}_4$, the maximum total nitrate reductase activities attained by these cultures of depleted organisms were always considerably less than the highest (not necessarily maximum) values previously observed with cultures of undepleted organisms growing under similar conditions (Wainwright & Pollock, 1949). It therefore seemed possible that the factor which limited the development of nitrate reductase activity by nitrogen-depleted cells might be a limiting rate of *de novo* synthesis of amino acids. In support of such an interpretation it was found that in the presence of an acid hydrolysate of casein ('Bacto Casamino acids') there was a marked increase in the total activity of the culture (Fig. 3).

Other possible interpretations of the limited increase in nitrate reductase activity and the rapid attainment of a maximum total activity in cultures of previously depleted cells were considered. The increase in enzyme activity was totally inhibited in the presence of 40 μg . chloramphenicol/ml., indicating that the process was indeed one of protein metabolism (Gale & Folkes, 1953, 1954; Wisseman, Smadel, Hahn & Hopps, 1954).

Nitrite did not inhibit the increase in nitrate reductase activity when added in a concentration of 0.001M and was only slightly inhibitory at a concentration of 0.01M (the maximum concentration which could accumulate during normal

induction). Thus, the absence of a continued rise in the total activity of the culture was not due to the accumulation of toxic concentrations of nitrite.

The induced cells showed no detectable nitrite reductase activity. Thus, the attainment of a maximum total nitrate reductase of the culture was not an artefact resulting from significant reduction of nitrite during the assays of nitrate reductase.

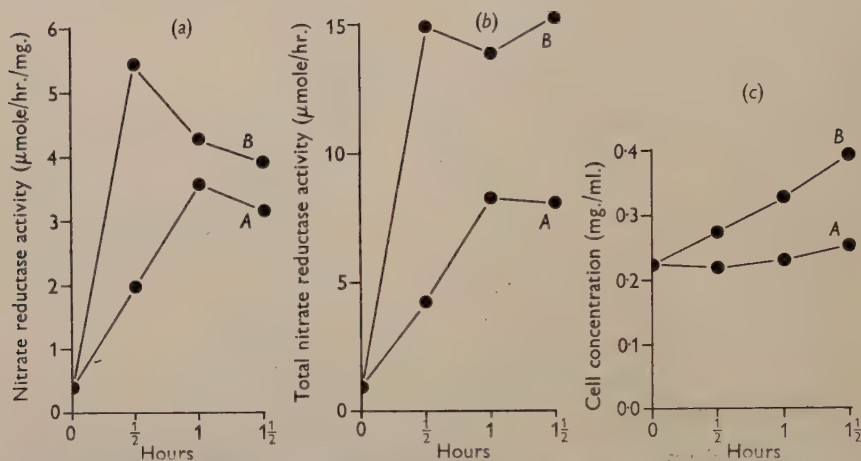


Fig. 3. Influence of casein hydrolysate (0.2%, w/v) on nitrate reductase formation by nitrogen depleted *Escherichia coli* 1433 induced with nitrate (0.01 M). (a) Nitrate reductase activities/mg. dry wt. of cells; (b) total nitrate reductase activities of cultures; (c) growth curves. A, without casein hydrolysate; B, with casein hydrolysate.

It seemed possible that the period of depletion, though adequate to deprive the cells of some key amino acid essential for β -galactosidase formation, was inadequate to deplete all nitrogenous reserves and that more prolonged starvation would eliminate the ability to form nitrate reductase. We therefore tested the ability of the cells to form enzyme when incubated anaerobically with nitrate both before exhaustion of the nitrogen source in the parent culture and at various intervals after the cessation of growth. The results of one such experiment are given in Fig. 4. Although complex, they are very different from those to be expected if more prolonged starvation had effected a greater decrease in the amount of available endogenous nitrogen reserves. Indeed, the most significant feature of these data is the inverse correlation between the total amount of enzyme formed (Fig. 4b) and the extent of the increase in cell mass after addition of nitrate inducer (Fig. 4c).

An alternative interpretation of the early cessation of increase in total nitrate reductase activity of the culture would be that the measured enzyme activity was not limited by the amount of enzyme protein present in the cells, but by the available quantity of some other accessory factor of the system. This possibility could not be tested directly, for the enzyme system has not yet been isolated from *Escherichia coli* and there are reasons for believing that the system may differ from the nitrate reductase system of

*Neurospora crassa** (Nicholas & Nason, 1954). However, the same general picture was obtained with glucose as H-donor in the assays (at 45° to prevent further adaptation) in place of formate. Yet the data of Fig. 4*b* and *d* indicate that the measured nitrate reductase activities of depleted cells are more likely to be affected by deficiency of co-factors when glucose is used as H-donor, for the adaptation curves (Fig. 4*d*) show no indication of a progressive increase in activity with increased period of depletion.

The maximum total nitrate reductase activity of the culture was not limited by deficiencies of either iron or molybdenum.

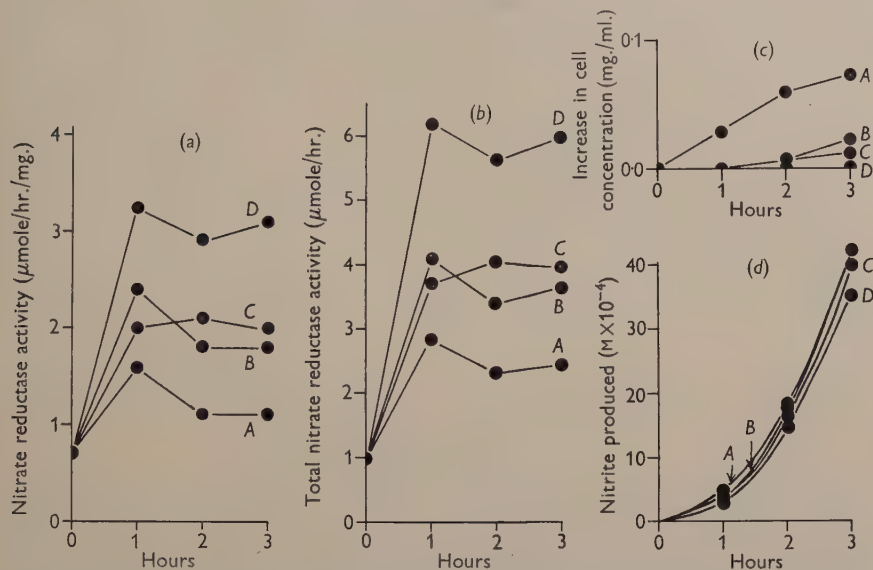


Fig. 4. Influence of duration of depletion period upon nitrate reductase formation by nitrogen depleted *Escherichia coli* 1433 induced with nitrate (0.01M). (a) Nitrate reductase activities/mg. dry wt. of cells; (b) total nitrate reductase activities of cultures; (c) growth curves (expressed as further increase during induction); (d) adaptation curves. Induction: A, 1½ hr. before exhaustion of nitrogen reserves; B, 1½ hr.; C, 3 hr.; D, 4½ hr. after depletion of reserves.

Tetrathionate reductase adaptation in nitrogen-depleted cells

When depleted cells were incubated anaerobically with tetrathionate and glucose (as energy source) there was a marked and immediate increase in tetrathionate reductase activity (Fig. 5). There was no increase in cell mass and, in fact, the optical density of the culture decreased slightly (Fig. 5*c*). The possibility of nitrogen sources being made available for enzyme synthesis by cell lysis cannot be excluded. However, the decrease in optical density, which

* Assays of crude preparations of the nitrate reductase of *Escherichia coli* 1433 by the method recently described by Nicholas & Nason (1955) usually gave values less than 1% of those obtained with reduced methylene blue as H-donor. Preliminary evidence suggests that a derivative of menodione is an essential co-factor of the enzyme (Wainwright, *Biochim. biophys. Acta*, in the press).

corresponded to the lysis of *c.* 25 μg . dry wt. cells/ml., also occurred in all anaerobic control cultures (e.g. Fig. 1). Yet, in the presence of lactose there was no synthesis of β -galactosidase, either in the presence or absence of tetrathionate (Fig. 1). Further, tetrathionate appeared to stimulate rather than inhibit β -galactosidase formation when $(\text{NH}_4)_2\text{SO}_4$ was added in concentrations as small as 0.0005% (w/v). Thus, tetrathionate reductase was formed either in the total absence of extraneous nitrogen sources, or, at least, in response to the presence of concentrations of nitrogen sources inadequate to support β -galactosidase formation. The adaptation process was markedly stimulated by the addition of $(\text{NH}_4)_2\text{SO}_4$. Chloramphenicol, at 40 μg /ml., totally inhibited induced tetrathionate reductase formation.

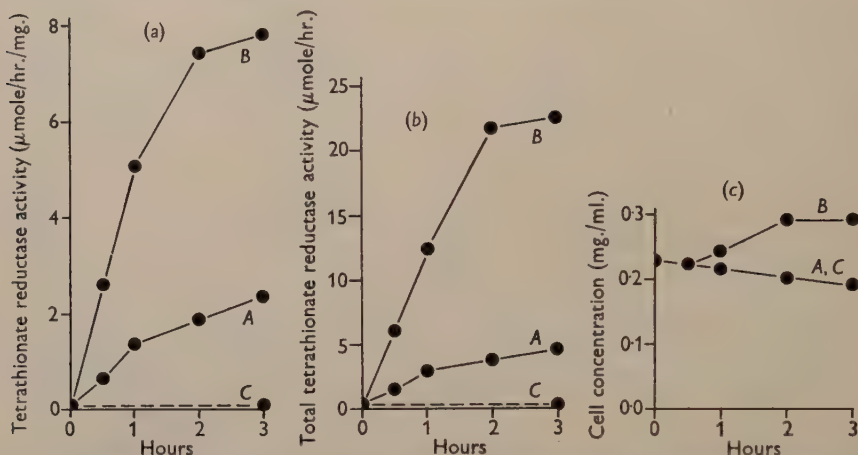


Fig. 5. Influence of $(\text{NH}_4)_2\text{SO}_4$ (0.005%, w/v) on tetrathionate reductase formation by nitrogen depleted *Escherichia coli* 1433 induced with tetrathionate (0.01 M). (a) Tetrathionate reductase activities/mg. dry wt. cells; (b) total tetrathionate reductase activities of cultures; (c) growth curves. A, without $(\text{NH}_4)_2\text{SO}_4$; B, with $(\text{NH}_4)_2\text{SO}_4$; C, control without tetrathionate or $(\text{NH}_4)_2\text{SO}_4$.

DISCUSSION

It is clear that the nitrogen requirements for induced formation of β -galactosidase by intact organisms of *Escherichia coli* 1433 differ from those for formation of the nitrate and tetrathionate reductases even with a single population of organisms (e.g. Figs. 1, 2). β -Galactosidase is not produced unless an external source of nitrogen is present, and even then there is an appreciable delay between the addition of lactose inducer and the appearance of induced enzyme. Further, this delay is not related in any simple manner to the lag period in the over-all synthesis of protein (Fig. 1). In contrast, the tetrathionate reductase system is formed without appreciable delay and in the absence of detectable nitrogen sources (Fig. 5). The possibility of nitrogen sources being made available by cell lysis has not been eliminated, but the extent of lysis which occurred during induction with tetrathionate was no greater than when cells were treated anaerobically with lactose. In the case of the nitrate

reductase system the inducing nitrate itself served as a nitrogen source. However, the absence of any appreciable lag in enzyme formation (Fig. 2) is in marked contrast to the result obtained for β -galactosidase.

These variations in nitrogen requirements may be manifestations of quantitative differences in the efficiencies with which homologous specific components of the various 'enzyme-forming systems' can compete for limiting amounts of amino acids (Wainwright, 1950). Alternatively, it seems possible that the cells can be depleted of all nitrogen sources which can serve as 'precursors' of β -galactosidase but yet contain materials which can be utilized for the synthesis of the reductases. If the latter be the case, the data obtained in this study would indicate that the process of enzyme synthesis consists of at least two phases: (1) *de novo* synthesis of 'precursor', which could only occur if either the depleted cells were supplied with an excess of pre-formed amino acids (Fig. 3), or if they were unable to use their own metabolic products for increase in cell mass (Fig. 4); (2) conversion of 'precursor' into active enzyme, which could occur in the presence of very low concentrations of available nitrogen source. The data do not permit discrimination between the two alternative types of interpretation. However, they do demonstrate a major difference between various 'enzyme-forming systems', at least in the intact organisms.

We are indebted to Messrs Parke Davis and Co. Ltd., Walkerville, Ontario, for a generous gift of pure chloramphenicol.

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Tetrazolium Reduction as a Means of Differentiating *Streptococcus faecalis* from *Streptococcus faecium*

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SUMMARY: Tetrazolium (2:3:5-triphenyltetrazolium chloride) was used in a glucose nutrient medium to show the considerable differences in reducing properties between some of the Lancefield group D streptococci. The ability to reduce tetrazolium to formazan, in a glucose-containing medium, at an initial pH 6.0, distinguishes strains of *Streptococcus faecalis* from those of *S. faecium* (Orla-Jensen) which for many years have been classified in the same species as *S. faecalis*. Differences in reducing properties correspond with many other tests now used to separate these species. Measurements of the oxidation-reduction potentials in growing cultures showed that *S. faecalis* produced an E_h value about 150 mV. lower than *S. faecium*. Confirmation of the differences in reducing properties of these species was obtained by testing 68 freshly isolated strains of *S. faecium* and 34 strains of *S. faecalis*.

The problem of differentiating the streptococci within the Lancefield group D has received considerable attention in the past few years because many of these organisms are important clinically and also because their unusual tolerance of salt, temperature and acid conditions renders them important in food spoilage. Considerable advances have been made in defining the species concerned, by correlating physiological reactions with serological types (Skadhauge, 1950; Seelemann & Carstens, 1951; Sharpe & Shattock, 1952). A case has been made for the re-introduction of the species *Streptococcus faecium* (Orla-Jensen, 1919) as a species separate from *S. faecalis* and its haemolytic and proteolytic variants *zymogenes* and *liquefaciens* (Shattock, 1955).

Streptococcus faecalis and *S. faecium* share the characteristics usually described as the 'Sherman criteria' for the enterococcus group (Sherman, 1937), but they may be distinguished by certain other properties (see Table 1). The necessity for distinguishing between these two species may be briefly explained as follows. (1) Both organisms have been isolated in human infections, but Skadhauge (1950) suggests that there may be a difference in the sites of infections with the two organisms. (2) Faecal streptococci have been implicated in a number of outbreaks of food-poisoning (Dack, 1944), and it is not yet known whether one or more types are responsible. (3) Although both species have been isolated in food spoilage, e.g. from canned hams, there is a suggestion that one (*S. faecalis*) may be indicative of poor hygiene, as this organism is mainly found in human faeces, whilst the other organism may get into the meat from the gut of the animal concerned, in this case, the pig (Ingram & Barnes, 1955; Barnes & Ingram, 1955). (4) *S. faecalis* is used as an indicator organism for water pollution.

Skadhauge (1950) and Barnes & Ingram (1955) found that there was a difference in the reducing properties of these two species as shown in litmus milk: *Streptococcus faecalis* usually produces reduction, acid, and clotting in 24 hr., but *S. faecium* shows less reduction and sometimes only acid production. The other two species in the Lancefield group D, *S. durans* and *S. bovis*, do not possess strong powers of reducing litmus milk, and usually cause only acidity or acidity and clotting.

Table 1. *The differentiation of Streptococcus faecalis from S. faecium*

S. faecalis and *S. faecium* share the following characteristics (Sherman criteria):

- Lancefield group D.
- Growth in the presence of 40 % bile.
- Fermentation of glucose, lactose and maltose.
- Growth at 10° and 45°.
- Growth at pH 9.6 and in the presence of 6.5 % NaCl.
- Survival at 60° for 30 min.
- Growth in Sherman's methylene blue milk (0.1 %).

They differ as follows:

<i>S. faecalis</i> , <i>S. faecalis</i> var. <i>zymogenes</i> and <i>S. faecalis</i> var. <i>liquefaciens</i>	<i>S. faecium</i>
A number of serological types.	Several serological types but distinct from the <i>S. faecalis</i> types.
Growth in the presence of 1/2500 potassium tellurite.	Inhibited by 1/2500 potassium tellurite.
Strong fermentation reactions; mannitol and sorbitol always fermented, arabinose seldom.	More variable fermentation reactions, particularly with mannitol; arabinose always fermented, sorbitol seldom.
Strong reduction in litmus milk prior to acid and clot.	Less reduction of litmus milk and sometimes no reduction, only acidity.
Active production of tyrosine decarboxylase.	Some strains are active producers of tyrosine decarboxylase, whilst others do not produce it.

2:3:5-Triphenyltetrazolium chloride ('tetrazolium' subsequently) has often been used recently as a redox indicator. It is colourless in the oxidized form and is reduced to the insoluble red triphenylformazan in the presence of suitable reducing substances or systems. The E'_0 was shown by Jerchel & Mohle (1944) to be -0.08 V. at pH 6.72. Laxminarayana & Iya (1953) found that certain unnamed strains of faecal streptococci varied in their ability to reduce tetrazolium. It was decided to adapt some of their methods to study tetrazolium reduction by known species of Lancefield group D streptococci, in particular *Streptococcus faecalis* and *S. faecium*.

METHODS

Estimation of tetrazolium reduction by faecal streptococci

Culture medium (subsequently called TG medium). The composition of the medium was (% w/v): peptone (Evans's), 1; Lab Lemco, 1; NaCl, 0.5; glucose, 1; distilled water; 2:3:5-triphenyltetrazolium chloride to give 375 µg/5 ml. medium. In the experiments described below the initial pH value was varied between 6.0 and 7.6.

The medium containing the peptone, Lemco and salt was adjusted to the required pH value and autoclaved at 15 lb./sq.in. for 20 min. in 95 ml. lots. After autoclaving, 5 ml. of a 20% (w/v) glucose solution (sterilized by autoclaving at 10 lb./sq.in. for 10 min.) were added together, with 0.75 ml. of a 1% solution of the tetrazolium salt. (The tetrazolium solution was sterilized by steaming for 30 min.). The TG medium was then aseptically pipetted in 5 ml. lots into 1 oz. screw-capped bottles.

Cultural conditions. The TG medium (5 ml.) was inoculated with 0.025 ml. of an 18 hr. broth culture to give $c. 1 \times 10^7$ organisms/ml. The inoculated bottles were incubated for 24 hr. at 37° and the reduced tetrazolium (insoluble red formazan) was extracted as follows.

Extraction of formazan from cultures. A culture (5 ml.) was shaken successively with 5, 2 and 2 ml. of *n*-butanol to extract all the formazan. The butanol extracts were withdrawn with a Pasteur pipette into marked centrifuge tubes and the volume made to 10 ml. with butanol. The solution was mixed and centrifuged to obtain a clear extract. The colour intensity of the extract was measured in an EEL. (Evans Electroselenium Ltd.) colorimeter with filter no. 625. The percentage reduction of tetrazolium was calculated by reference to a standard curve (see below) on the assumption that it was directly proportional to the amount of formazan produced.

Errors of the method. Care must be taken that the tetrazolium solution is freshly prepared and stored in the dark. In media where considerable reduction has taken place (e.g. with *Streptococcus faecalis*) sufficient formazan to give a visible colour adheres to the organisms during growth and cannot be removed by the butanol extraction. However, even in these cases virtually all the formazan may be recovered (see Fig. 2), the quantity adsorbed evidently being insufficient to interfere with the estimation.

Standard curve for estimation of tetrazolium reduction. Bottles of TG medium (5 ml., pH 7.0) containing 375, 300, 225, 150 or 75 µg. tetrazolium salt/bottle were treated with 1 ml. *N*-NaOH and several mg. ascorbic acid to reduce all the tetrazolium present (Fairbridge, Willis & Booth, 1951). The bottles were left for 30 min. and then adjusted to about pH 4.5 with *N*-HCl (to give a pH for extraction comparable to that obtained after the growth of the streptococci). The formazan was extracted from the broths with *n*-butanol (as above) and a calibration curve (Fig. 1) obtained by relating the colorimeter reading to the initial concentration of tetrazolium salt (when completely reduced to formazan).

Measurement of growth. The total number of organisms present was estimated either by a direct microscopical count or by a turbidimetric method using the EEL nephelometer, a standard curve having been made by relating direct microscopical count to nephelometer reading.

E_h measurements. The redox potentials in the broth cultures were measured by the method described by Hewitt (1950). In this case tall beakers containing 25 ml. medium were fitted with bungs carrying the sampling tube, the platinum electrode and a saturated KCl agar bridge connected to the calomel half-cell. The platinum electrodes were sterilized separately by leaving in

70% (v/v) ethanol overnight and then rinsing with sterile water and drying with sterile cotton-wool. In these experiments the object was not to eliminate all the air but to imitate as far as possible the conditions existing in TG medium in the 1 oz. bottles used for routine tests. The inoculum for these experiments was 1% (v/v) of an overnight broth culture so that the major part of the experiment was concluded in 8–10 hr., although readings were also made after 24 hr. When tetrazolium estimations were made on duplicate control solutions in 1 oz. bottles the inoculum was also increased to 1% (v/v) but the cultures were incubated for 24 hr. in the usual manner.

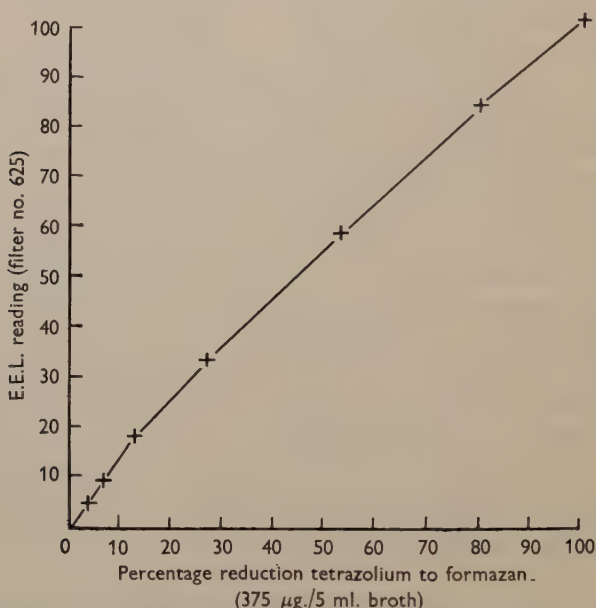


Fig. 1. Calibration curve relating the percentage reduction of 375 µg. of tetrazolium (as formazan) to the reading on the EEL colorimeter.

RESULTS

For a preliminary survey of the ability of group D streptococci to reduce tetrazolium to formazan, named strains were obtained from Dr P. M. Frances Shattock (University of Reading) and Dr M. Elisabeth Sharpe (National Institute for Research in Dairying). The tests were carried out by the method described above; the pH value of the TG medium was 7.2–7.4. At the same time a comparison was made with the reaction in litmus milk. The results are given in Table 2. The three strains of *Streptococcus faecalis* showed over 80% reduction of tetrazolium to the insoluble red formazan and produced rapid reduction, acid and clot in litmus milk. The five strains of *S. faecium* produced less than 45% reduction of tetrazolium, and litmus milk was only partially reduced in 24 hr. *S. bovis* and *S. durans* reduced the tetrazolium by less than 10%, and produced little change in litmus milk in 24 hr.

These results encouraged further experiments with tetrazolium and freshly isolated strains of *Streptococcus faecium* and *S. faecalis*. The identifications were based on the properties shown in Table 1. Over a period of several months 68 strains of *S. faecium* and 34 strains of *S. faecalis* were tested in TG medium (pH 6.8–7.0) and the differences in their reducing powers was found to be quite characteristic. These results are shown in Fig. 2. Only 6 strains of *S. faecium* gave over 50% reduction of tetrazolium, whilst 2 strains of *S. faecalis* gave less than 50% reduction.

Table 2. *A comparison of the reaction in litmus milk with the reduction of tetrazolium for several species of Lancefield group D streptococci*

Species	Litmus milk			TG medium	
	6 hr.	24 hr.	3 days	Total count × 10 ⁹ /ml. after 24 hr.	% reduction to formazan
<i>S. faecalis</i> (N. 83)	R*	Rc	RAC	2.0	86
<i>S. faecalis</i> var. <i>liquefaciens</i> (GB. 122)	r	RCAIq.	RCAIq.	2.0	91
<i>S. faecalis</i> var. <i>zymogenes</i> (N. 37)	R	RAC	RAC	2.0	85
<i>S. faecium</i> 2766/2	nc	A	ARC	4.0	24
<i>S. faecium</i> 2767	nc	Ar	ARC	2.5	31
<i>S. faecium</i> 2678	nc	Ar	ARC	3.0	35
<i>S. faecium</i> S. 748	r	Ar	ARC	3.0	39
<i>S. faecium</i> 2766/1	nc	Ar	ARC	3.0	43
<i>S. durans</i>	nc	A	AR	2.5	10
<i>S. bovis</i>	nc	nc	A	2.5	9

* nc, no change; A, acid; r, some reduction; R, strong reduction; c, slight clot; C, clot, Iq., liquefaction.

Relationship of redox potential to tetrazolium reduction

In order to determine whether differences in tetrazolium reduction were related to different reducing powers of these organisms, measurements were made of the actual oxidation-reduction potentials of cultures in TG medium. Tests which were carried out separately in tetrazolium glucose medium of different initial pH values showed that after the first few hours the growth rates and pH changes were comparable for strains of *Streptococcus faecalis*, *S. faecium*, *S. durans* and *S. bovis*, as shown in Table 3. Thus any recorded ultimate differences in redox potential would be a reflexion of different reducing properties of the organisms concerned, rather than a function of the pH value of the culture or of the numbers of organisms present.

In the redox potential experiments described below only the pH changes are recorded as an index of the growth of the organisms, as it was difficult to obtain homogenous samples for total count without disturbing the redox potential of the medium. Samples were, however, removed at the end of the experiment and these, together with a few samples taken at intervals, showed that the counts were not significantly different from those recorded in Table 3.

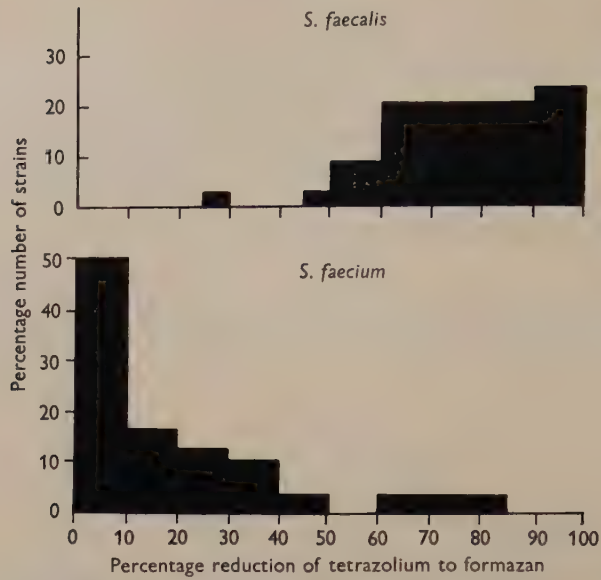


Fig. 2. A comparison of the percentage reduction of tetrazolium to formazan by strains of *Streptococcus faecalis* and *S. faecium*.

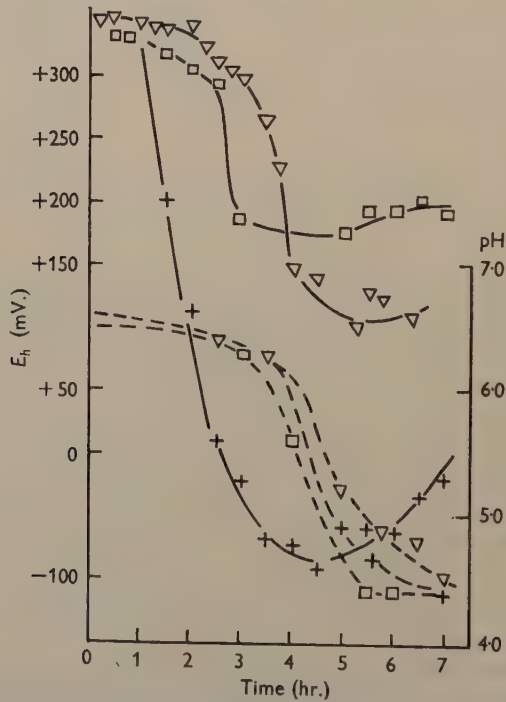


Fig. 3. Changes in the redox potential and pH during the growth of *Streptococcus faecalis*, *S. faecium* and *S. bovis*.

+ — +, E_h } *S. faecalis* ▽ — ▽, E_h } *S. faecium* □ — □, E_h } *S. bovis*.
 + - - - +, pH } *S. faecalis* ▽ - - - ▽, pH } *S. faecium* □ - - - □, pH } *S. bovis*.

The changes in E_h and pH were followed for 7–8 hr. at 37° when beakers of TG medium (pH 6.5–6.6) were inoculated with 1% (v/v) of an overnight broth culture of *Streptococcus faecalis* (N. 88), *S. faecium* (S. 748) and *S. bovis* (S. 194). The results are shown in Fig. 3. The lowest potential reached in the *S. faecalis* culture was about E_h –90 mV., that of *S. faecium* was +90 mV.

Table 3. *The effect of initial pH on the growth and total population in glucose broth of Streptococcus faecalis, S. faecium, S. bovis and S. durans*

		Initial pH							
		6.1		6.6		7.1		7.4	
		Total count		Total count		Total count		Total count	
	Time (hr.)	pH	$\times 10^9/\text{ml.}$	pH	$\times 10^9/\text{ml.}$	pH	$\times 10^9/\text{ml.}$	pH	$\times 10^9/\text{ml.}$
<i>S. faecalis</i>	4	5.6	0.5	6.5	0.7	6.9	0.7	7.0	1
	6	4.5	2.5	4.5	4.0	4.5	5.5	4.5	5.5
	24	4.1	4.0	4.1	5.5	4.1	6.5	4.1	6
<i>S. faecalis</i> var. <i>liquefaciens</i>	4	5.8	0.5	6.4	0.9	6.8	1.0	6.9	0.9
	6	4.5	2.0	4.5	3.5	4.5	4.5	4.5	4.5
	24	4.1	3.0	4.1	4.5	4.1	5.0	4.1	5.0
<i>S. faecalis</i> var. <i>zymogenes</i>	4	5.8	0.5	6.4	0.7	6.4	1.0	6.9	0.9
	6	4.5	2.5	4.5	4.0	4.5	5.0	4.5	5
	24	4.1	4.0	4.1	5.0	4.1	6.0	4.1	6
<i>S. faecium</i> 2766/1	4	6.0	0.4	6.5	0.4	6.9	0.5	7.1	0.4
	6	4.5	3.5	4.5	5.0	4.5	5.5	4.5	5.5
	24	4.1	4.0	4.1	5.5	4.1	6.5	4.1	6
<i>S. faecium</i> S. 748	4	5.9	0.4	6.5	0.5	6.9	0.5	7.1	0.5
	6	4.5	3.0	4.5	4.5	4.5	5.5	4.5	5.5
	24	4.1	4.0	4.1	5.5	4.1	6.0	4.1	6.0
<i>S. faecium</i> 2678	4	6.0	0.4	6.4	0.5	6.9	0.6	7.0	0.6
	6	4.5	3.5	4.5	5.0	4.5	5.5	4.5	5.9
	24	4.1	4.5	4.1	5.5	4.1	6.0	4.1	6.0
<i>S. durans</i>	4	5.9	0.4	6.4	0.6	6.9	0.6	7.0	0.6
	6	4.5	3.0	4.5	4.4	4.5	5.0	4.5	5.5
	24	4.1	4.0	4.1	5.0	4.1	6.0	4.1	6
<i>S. bovis</i>	4	6.1	0.3	6.5	0.4	7.0	0.4	7.3	0.4
	6	4.5	1.5	4.5	3.0	5.7	2.5	5.8	2.5
	24	4.1	3.5	4.1	5.5	4.1	6.0	4.1	5.0

and *S. bovis* +180 mV. Thus the reducing conditions were much greater in the *S. faecalis* culture than in either the *S. faecium* or *S. bovis* cultures. This experiment was repeated on several occasions, and the marked difference between *S. faecalis* and *S. faecium* always obtained. Although the *S. faecium* strain used was usually more reducing than *S. bovis*, occasionally it showed little difference from *S. bovis* in the TG medium. As this investigation was concerned mainly with the differences between *S. faecium* and *S. faecalis* detailed investigations of the relationship of *S. faecium* to *S. bovis* were not made.

Effect of changes in the medium on tetrazolium reduction

Alteration in the initial pH value of the glucose tetrazolium medium. The initial value of samples of TG medium was adjusted to 6.1, 6.6, 7.1 and 7.4 respectively, and inoculated with a number of strains of *Streptococcus faecalis*, *S. durans* and *S. bovis*. The results obtained from a number of experiments are shown in Fig. 4 where tetrazolium reduction is related to the initial pH value of the

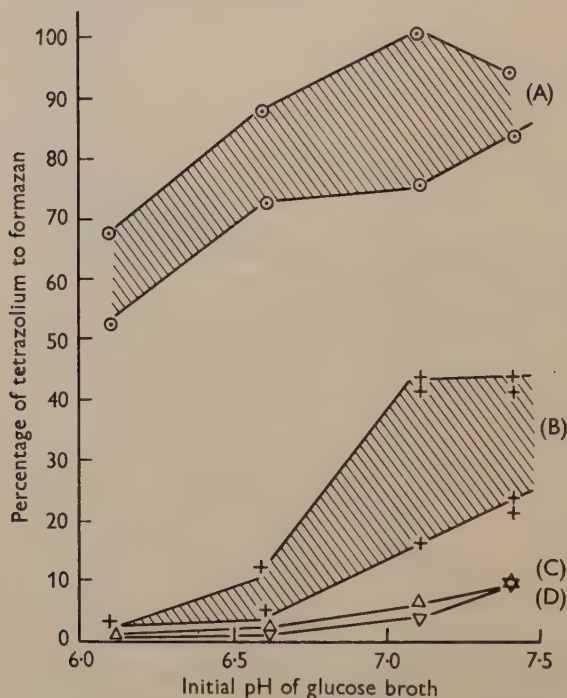


Fig. 4. The effect of the initial pH of glucose broth on the reduction of tetrazolium to formazan by (A) *S. faecalis*, (B) *S. faecium*, (C) *S. durans* and (D) *S. bovis*.

medium. With the strains of *S. faecalis* there was over 50% reduction of tetrazolium to formazan at all pH values, whilst with *S. durans* and *S. bovis* there was less than 10%. In the case of *S. faecium* the initial pH value considerably influenced the amount of formazan produced during growth. It can be seen that in order to get the maximum differentiation between *S. faecium* and *S. faecalis* the test should be carried out at pH 6.0–6.2. With a strain of *S. faecium* an attempt was made to correlate the amount of formazan produced in the media of different initial pH values with the lowest redox potential obtained in that particular medium. Although there was no absolute correlation the lowest potentials recorded varied from $E_h + 210$ mV. in medium of initial pH 6.0 to $E_h + 120$ mV. when the initial pH was 7.6. In the same experiment the reduction of tetrazolium varied from less than 2.0 to 28%.

Effect of alterations in the composition of the medium. The effect on tetrazolium reduction of growing the organisms in media where there was only a slight change in pH value was tested in the following manner. Samples of Hartley's tryptic digest broth containing the usual concentration of tetrazolium were adjusted to pH 6.0, 6.4, 6.8 and 7.2. The broths were inoculated with strains of *Streptococcus faecalis*, *S. faecium* and *S. bovis*, and incubated for 24 hr. In every case there was more than 50% reduction of tetrazolium to formazan, i.e. no difference was detected in the reducing properties of the organisms. In order to explain this a redox potential experiment was set up where TG medium and digest broth at pH 6.1–6.2 were compared under

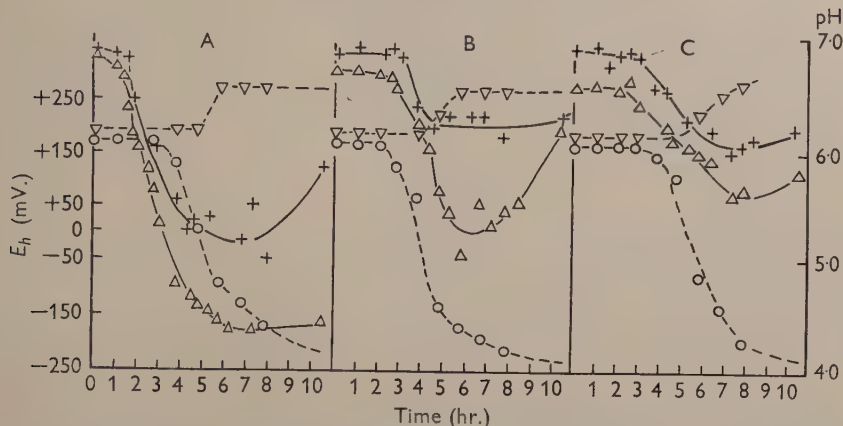


Fig. 5. Changes in E_h and pH of (A) *S. faecalis*, (B) *S. faecium* and (C) *S. bovis* when grown in glucose broth and digest broth.

+ — +, E_h } in glucose broth \triangle — \triangle , E_h } in digest broth.
 ○ — ○, pH } ∇ — ∇ , pH }

identical conditions, with inocula of the three different species. Changes in redox potential and pH are shown in Fig. 5. In the digest media the pH value rose and the actual oxidation-reduction potentials were much lower, by 80 or more mV., than those reached in the glucose media. Even in the case of *S. bovis* the E_h of the digest broth was as low as +74 mV. so that all three species then reduced the tetrazolium.

Reduction of tetrazolium at pH 6.0. From the above experiments it was apparent that the best medium to use for obtaining the maximum differentiation between *Streptococcus faecalis* and *S. faecium* was a TG medium of initial pH 6.0–6.2, as the redox potentials reached in this medium were such that there was no reduction with cultures of *S. faecium* but over 50% reduction with *S. faecalis*. In view of a statement by Jámboř (1954) that at pH values below 6.0 tetrazolium is partially reduced to a colourless compound, it was decided to estimate the residual unreduced tetrazolium in the cultures to see whether all the initial tetrazolium could be accounted for.

Samples of TG medium were inoculated in quadruplicate with *Streptococcus faecalis*, *S. faecium* or *S. bovis*, and incubated for 24 hr. Two bottles were taken

for the estimation of formazan in the usual manner. The other two bottles were treated with 1 ml. of N-NaOH followed by a few mg. ascorbic acid to reduce all the residual tetrazolium to formazan. The broths were then acidified by the addition of 1 ml. of N-HCl and the total formazan extracted from the solution as before. The results are shown in Table 4. With *S. faecium* and *S. bovis* almost all of the tetrazolium was recovered from the medium so that it had not been reduced by the organisms. In the case of *S. faecalis* about 30% of the tetrazolium was not recovered; the reduction of tetrazolium had

Table 4. *The reduction of tetrazolium by Streptococcus faecalis, S. faecium and S. bovis in media of initial pH 6.0*

	% of tetrazolium reduced to formazan	Residual unreduced tetrazolium (%)	% of tetrazolium unaccounted for in the medium
Control solution	0	100	0
<i>S. faecalis</i> (N. 83)	59 } 45 } 54 59 }	8 } 19 } 14 16 }	32
<i>S. faecalis</i> var. <i>zymogenes</i>	46 } 54 } 50	22 } 27 } 25	25
<i>S. faecium</i> (S. 748)	1.5 } 0.5 } 1.0	90 } 92 } 91	8
<i>S. bovis</i>	0.5 } 0.5 } 0.5	97.5 } 97.5 } 97.5	2

either resulted in a mixture of the formazan and a colourless compound, or the formazan had been reduced further. It would therefore be unwise to attempt quantitative estimations of tetrazolium reduction in culture media with an initial pH of 6.0; but qualitatively, sufficient formazan is produced by strains of *S. faecalis* to give a bright red colour when extracted with butanol as opposed to an almost colourless solution with strains of *S. faecium*.

Qualitative methods for the separation of Streptococcus faecalis and S. faecium

As a result of the above experiments the following media were developed to assist in the separation of *Streptococcus faecalis* and *S. faecium*.

(a) *TG medium at pH 6.0-6.1*. This medium was prepared as described above, but was adjusted to an initial pH of 6.0-6.1. Instead of extracting the formazan after overnight incubation with the test organisms, the cultures were shaken with 5 ml. of *n*-butanol and allowed to settle. Positive reactions were indicated by a bright red butanol layer whilst negative reactions were either colourless or very pale pink. About 70 of the isolates of *Streptococcus faecalis* and *S. faecium*, which had previously been tested in TG medium at pH 6.8-7.0 with the results shown in Fig. 2, were re-tested in the TG medium at pH 6.0 by this qualitative method. The strains could now be divided easily into *S. faecalis* with the bright red reaction and *S. faecium* with a colourless

reaction. The only exceptions were those few strains which had previously been atypical—in the case of *S. faecalis* having a low reducing power, and with *S. faecium* having an exceptionally high one.

(b) *Tetrazolium* agar for isolating *Streptococcus faecalis* and *S. faecium*. A useful extension of the methods described above was the preparation of a solid medium for separating these two organisms. This medium had the same composition as TG medium but the concentration of tetrazolium was increased to 0.01 %, and it was solidified by addition of 1.2 % (w/v) New Zealand agar; the pH value was adjusted to 6.0–6.1. The agar, peptone, Lemco and salt was autoclaved at 15 lb./sq.in. for 20 min. in 95 ml. lots and the glucose and tetrazolium solutions added immediately before the plates were poured. The plates were streaked with cultures of the test organisms and incubated for 1 or 2 days at 37°. Isolated colonies of *Streptococcus faecalis* were deep red, whilst those of *S. faecium* were colourless. When cultures of *S. faecalis* were mixed with *S. faecium* it was quite easy to separate the two species again by this method.

DISCUSSION

The results given in this paper emphasize the great differences in the reducing powers of the two related species, *Streptococcus faecium* and *S. faecalis*, and substantiate the position of *S. faecium* (Orla-Jensen) as a species distinct from *S. faecalis* and its variants *zymogenes* and *liquefaciens*. Within the Lancefield group D, *S. faecium* appears to occupy an intermediate position between *S. faecalis* on the one side and *S. durans* and *S. bovis* on the other.

As there was a difference in E_h of over 100 mV. between the cultures of *Streptococcus faecalis* and *S. faecium* grown under the same conditions, it was possible to adjust the initial pH value of TG medium so that with *S. faecium* there was no reduction of tetrazolium during growth, whilst with *S. faecalis* the tetrazolium was almost entirely reduced.

This test with TG medium at pH 6.0 augments the other distinguishing tests given in Table 1. The advantage of using tetrazolium in such a test is that the production of the insoluble formazan is an irreversible reaction. This is important as it was observed in all the redox potential experiments with TG medium that the E_h rose rapidly before growth had ceased. Hewitt (1950) also found this with other streptococci, the rise in potential being attributed to the production of hydrogen peroxide. Hence, with a reversible redox indicator such as methylene blue, reduction is liable to be only temporary, making the test ambiguous.

In the experiments described above it is evident that the reduction of tetrazolium took place in cultures of a much higher redox potential than would be expected from the E_0 of –80 mV. at pH 6.72 given by Jerchel & Mohle (1944). From the information available it seems that once the potential falls below about $E_h + 150$ mV. reduction of tetrazolium begins.

The tetrazolium glucose agar medium at pH 6.0 has been found most valuable for distinguishing the colonies of *Streptococcus faecalis* from *S. faecium* and the other Lancefield group D streptococci.

Cooper & Ramadan (1955) showed that true *Streptococcus faecalis* organisms which strongly reduced litmus milk and also Janus Green were typical of human faeces. They isolated a number of strains from bovine and sheep faeces which they listed as atypical *S. faecalis* strains and which were shown to have low reducing powers. These organisms were probably similar to the *S. faecium* strains described in this paper. Unfortunately the concentration of potassium tellurite (1/5000) used by Cooper & Ramadan was too low to inhibit their atypical strains if indeed they were *S. faecium*. Skadhauge (1950) showed that 1/2500 tellurite was the optimal concentration for separating *S. faecalis* and *S. faecium*; at higher dilutions *S. faecium* would grow.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research. The experimental work was carried out with the technical assistance of Mr G. Ingram and Mr K. H. Robinson.

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Turbidity Changes in Bacterial Suspensions in relation to Osmotic Pressure

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SUMMARY: The turbidity of suspensions of various Gram-negative bacteria is affected by the tonicity of the suspending medium. The light extinction of the suspension increases in a nearly exponential fashion with the solute concentration and approaches a maximum at concentrations close to 0.15M-NaCl. The maximal optical response to tonicity changes varies widely in different bacterial species, ranging from 30 to 140 % increase in light extinction as compared with the turbidity of the suspension in distilled water. The turbidity changes seem to depend on the viability of the bacterial cells as they cannot be elicited in bacteria killed by various procedures. The influence of the described phenomenon on the determination of bacterial cell concentration by the customary turbidimetric methods is discussed and its application for the estimation of the number of viable cells is suggested.

In the course of a study of the effect of osmotic pressure on the respiration of certain micro-organisms, it was observed that the turbidity of bacterial suspensions changed with variations of the osmotic pressure of the suspending medium. Since to our knowledge such a phenomenon has not been described in bacteria before, an investigation into the factors governing these turbidity changes was undertaken.

METHODS

Culture media. The micro-organisms with the exceptions indicated below were grown on nutrient agar (Difco) slopes. For cultivation of *Corynebacterium xerosis* the nutrient agar was fortified with 1 % (w/v) maltose and 5 % (v/v) neutralized tomato juice. Five % (w/v) NaCl was included in the medium for the growth of halophilic organisms and it was supplemented with 0.1 % Tween 40 for *Mycobacterium phlei*. *Pasteurella pestis* was cultivated on brain heart infusion agar (Difco), *P. tularensis* on a 3 % (w/v) peptone (Difco) cysteine medium (Mager, Traub & Grossowicz, 1954), and *Clostridium botulinum* in a casein hydrolysate partially defined medium as described by Mager, Kindler & Grossowicz (1954).

Inoculum. For inoculation usually 0.1 ml. of a 24 hr. culture suspended in 5.0 ml. saline was used. Unless otherwise stated, the cultures were incubated at 37°.

Chemicals. All chemicals used were of reagent grade. The different biological preparations were purchased from commercial sources.

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Procedure for measuring the optical effect. The bacterial cells were washed twice with distilled water and the final aqueous suspension was adjusted to an optical density (E_w) of 0.20 as measured at 500 m μ . in the Coleman Junior spectrophotometer. The solute in the required concentration was added in 0.3 ml. volume to 3.0 ml. of the above suspension and the resulting extinction (E_s) was read after 1 min. and corrected for the dilution. In some experiments the Beckman spectrophotometer model D.U. was used (Corex cells, 1 cm. light path).

RESULTS

With increasing amounts of electrolytes or non-electrolytes added to suspensions of bacteria in distilled water, the light extinction increased gradually. The effect increased up to concentrations close to the range of isotonicity of these substances (Fig. 1). For a given solute concentration the percentage increase of light extinction, referred to the turbidity of the suspension in water, remained constant over a wide range of dilutions (from $E_{500} = 0.700$ to $E_{500} = 0.030$ in the Beckman spectrophotometer) and was independent of the wavelength used.

Survey of micro-organisms

Representatives of the different species of bacteria were assayed with NaCl or glucose for turbidity changes, as described under Methods. A scrutiny of the results, assembled in Table 1, shows that all the Gram-negative organisms examined exhibited the optical effect (O.E.) when tested with NaCl and most of them also in the glucose test; no appreciable O.E. could be demonstrated with any of the Gram-positive bacteria tested.

The magnitude of the effect (E') in percentage ($E' = \frac{E_s - E_w}{E_w} \times 100$, where E_w is the light extinction of the suspension exhibited in water, and E_s the extinction in the solute) varied with different Gram-negative organisms from 30 to 140. The high effect (E' from 100 to 140) exhibited by the different strains of *Pasteurella tularensis* made it especially suitable for quantitative studies of the phenomenon. In most experiments the avirulent strain S & D was used.

Solutes which produced optical effect (O.E.)

The osmotic nature of the O.E. was indicated by the finding that different substances, both electrolytes and non-electrolytes, elicited turbidity changes. Some solutes, such as urea, ethylurethane and glycols, which are known to be highly diffusible through biological membranes (Colländer, 1937; Danielli, 1952) and high molecular substances (inulin, plasma albumin) were ineffective (Table 2). As can be seen from Fig. 1, the mono-monovalent electrolytes tested exhibited, in equimolar concentrations, equal optical effects. With other solutes, however, the magnitude of O.E. (E') as compared with NaCl of equal molar strength, varied with the concentration. For 0.033M solutions the values obtained for the ratio E' solution/ E' NaCl were: Na_2SO_4 , 1.44; MgCl_2 , 1.80; glucose, 0.46; for higher concentration it approached unity, i.e. for 0.132M: Na_2SO_4 , 1.03; MgCl_2 , 1.83; glucose, 0.99.

Table 1. Test for optical effect in different micro-organisms

Age of culture: 18 hr. Conditions of growth as indicated in Methods. The o.e. of the halophilic micro-organisms *Micrococcus halodenitrificans* and *Vibrio costicus* represents the difference in light extinction exhibited in 1 % (w/v) and 7 % (w/v) NaCl solutions respectively.

	Optical effect in				Optical effect in		
	NaCl Glucose				NaCl Glucose		
(1) <i>Escherichia coli</i> strain 6	++	+		(20) <i>P. pestis</i> , strain H ₂	+	+	
(2) <i>E. coli</i> strain B	++	+		(21) <i>P. tularensis</i> , strain Vir	+++	nt	
(3) <i>E. coli</i> strain B/r	++	++		(22) <i>P. tularensis</i> , strain 176	+++	nt	
(4) <i>Aerobacter aerogenes</i>	+	nt		(23) <i>P. multocida</i>	++	nt	
(5) <i>Proteus vulgaris</i>	+	nt		(24) <i>P. pseudotuberculosis</i>	++	nt	
(6) <i>Alcaligenes faecalis</i>	++	++		(25) <i>Brucella suis</i>	++	+	
(7) <i>Salmonella schottmuelleri</i>	++	nt		(26) <i>B. abortus</i>	++	nt	
(8) <i>S. hirschfeldii</i>	++	+		(27) <i>B. melitensis</i>	++	nt	
(9) <i>S. paratyphi</i>	++	+		(28) <i>Malleomyces mallei</i>	++	nt	
(10) <i>S. typhosa</i>	++	nt		(29) <i>Corynebacterium xerosis</i>	—	—	
(11) <i>Shigella ambigua</i>	++	+		(30) <i>Lactobacillus arabinosus</i>	—	nt	
(12) <i>S. sonnei</i>	++	+		(31) <i>Mycobacterium phlei</i>	nt	—	
(13) <i>Micrococcus lysodeikticus</i>	—	—		(32) <i>Bacillus subtilis</i>	—	—	
(14) <i>Staphylococcus aureus</i>	—	—		(33) <i>B. anthracis</i>	—	nt	
(15) <i>Staphylococcus albus</i>	—	nt		(34) <i>Clostridium parabotulinum</i>	—	nt	
(16) <i>Streptococcus faecalis</i>	—	—		(35) <i>Vibrio cholerae</i> , 35	++	++	+
(17) <i>Neisseria perflava</i>	+	nt		(36) <i>V. paracholerae</i> (El-Tor)	++	nt	
(18) <i>N. catarrhalis</i>	++	nt		*(37) † <i>Micrococcus halodenitri-</i>	++	nt	
(19) <i>Pasteurella pestis</i> , 1122	++	nt		<i>ficans</i>			
				*(38) <i>V. costiculus</i>	++	nt	

— denotes turbidity changes less than 5 %; + denotes turbidity changes from 5 to 50 %; ++ denotes turbidity changes from 50 to 100 %; +++ denotes turbidity changes over 100 %; nt = not tested.

* Halophilic species kindly supplied by Dr W. L. Flannery, University of Maryland.

† This strain was Gram-negative by the staining procedure used (Burke, 1922).

Table 2. Effect of various solutes on optical effect

Solutes, unless otherwise indicated, were used in 0.5M concentration. The test organisms were: *Escherichia coli* (strains 6 and 5; 18 hr.) and *Pasteurella tularensis* (strain S & D; 24 hr.).

Solutes which produced the optical effect		Solutes which did not produce the optical effect
Sodium chloride	Ammonium citrate	Urea
Ammonium chloride	Sodium glutamate	Ammonium formate
Calcium chloride	Ammonium glutamate	Ammonium acetate
Magnesium chloride	Sodium pyruvate	Glycerol
'Tris' chloride	Ammonium pyruvate	Ethylene glycol
Histidine chloride	Sodium glycerophosphate	Propylene glycol
Sodium sulphate	Sodium phthalate	Inulin 2 % (w/v)
Ammonium sulphate	Ammonium phthalate	Bovine plasma albumin 1 % (w/v)
Manganous sulphate	Glycine	
Sodium nitrate	Glucose	
Ammonium nitrate	Galactose	
Sodium formate	Mannose	
Ammonium formate	Sucrose	
Sodium acetate	Inositol	
'Tris'-acetate	Sorbitol	
Histidine acetate	Mannitol	
Sodium citrate		

Influence of phase of growth and pH value

Under otherwise identical conditions the o.e. was found to be maximal with cells harvested at the end of the lag phase. The o.e. decreased during the logarithmic stage of growth and attained a constant level in the stationary phase (Fig. 2).

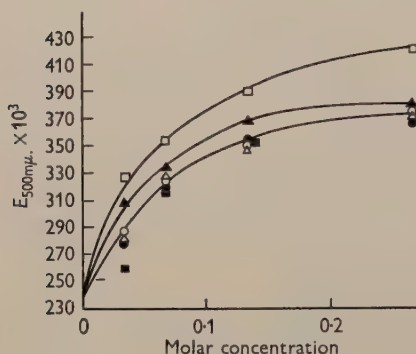


Fig. 1

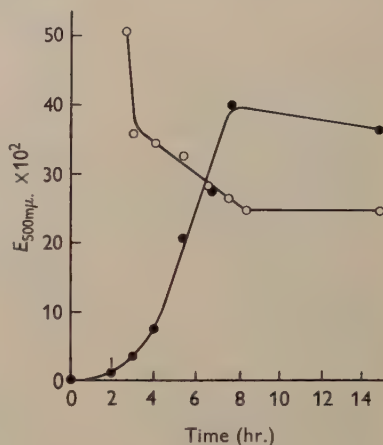


Fig. 2

Fig. 1. Changes in the light extinction of suspensions of *Pasteurella tularensis* with variation in the osmotic pressure of the suspending medium. Each Beckman cell contained the solutes in the concentrations indicated. Total volume 3.0 ml. Aqueous suspension of *P. tularensis* (strain S & D) was added and the turbidity changes followed with the time. The maximal values of the extinctions were recorded. ○—○, NaCl; ●—●, KCl; △—△, NaNO₃; ▲—▲, Na₂SO₄; □—□, MgCl₂; ■—■, Glucose.

Fig. 2. Phase of growth and optical effect: ○—○, percentage increase in light extinction (*E'*) on addition of NaCl (final concentration 0.5M) to an aqueous suspension of *Escherichia coli*; ●—●, growth curve of *E. coli* in nutrient broth. Optical effect was determined on samples taken at the times indicated.

In the different strains of *Pasteurella tularensis* tested, the period of incubation could be extended for several days (10–14) without any decrease in the o.e. being noticed. With other organisms, however, prolonged incubation resulted in a progressive diminution of the optical response of the bacteria to changes in osmotic pressure. As the medium used for the cultivation of *P. tularensis* contained unusually large amounts of buffer (Mager, Traub & Grossowicz, 1954), it was thought that the stability of the pH value in these cultures might account for the exceptional behaviour of this micro-organism. In fact, when the composition of the culture media used for other micro-organisms was modified in such a manner as to prevent large variations of pH value during growth, the decrease in the o.e. was considerably delayed (Table 3).

When washed cells were incubated in buffer solutions of different pH values the o.e. proved to be most persistent between pH 6 and 7 (Fig. 3). The similarity between the curve of Fig. 3 and the plate counts (cf. Winslow &

Falk, 1923) suggested that the o.e. depends on the viability of the organisms. This assumption was strengthened by the observation, that certain injurious treatments such as heating (Fig. 4) or alternate freezing and thawing (Table 4) reduced both the viability of the bacteria and the o.e. in nearly the same

Table 3. *Influence of variations of pH value in the culture medium on viable count and optical effect*

At the time of inoculation, the pH value of culture media was 6.8 in the two media tested.

Age of culture (hr.) Viable count (organisms/ml.) <i>E'</i> value* pH value	Phosphate buffer 1 % (w/v) Glucose 0 % (w/v)		Phosphate buffer 0.1 % (w/v) Glucose 2 % (w/v)	
	18	120	18	120
	2.6×10^8 50 7.3	2.2×10^8 50 7.4	8.5×10^7 80 4.8	8×10^6 10 4.5

* *E'*, percentage increase in light extinction (see page 70).

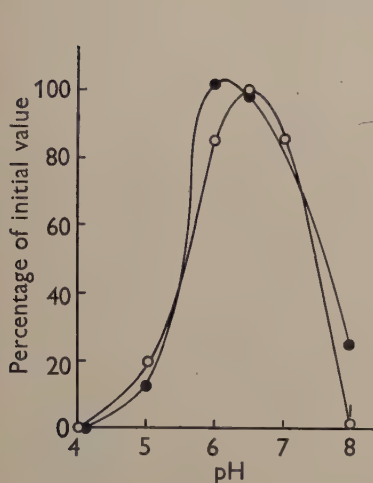


Fig. 3

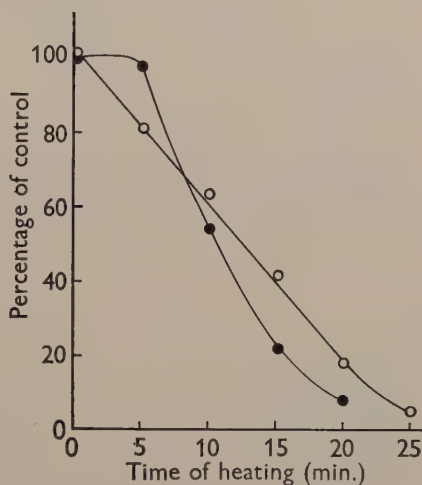


Fig. 4

Fig. 3. Effect of pH value on the viability and optical effect. *Escherichia coli* (strain 5; 20 hr.) was incubated with McIlvaine buffer (0.1 M) at various pH at 37°. After 17 hr. bacteria were washed and optical effect measured. ○—○, optical effect; ●—●, viable count.

Fig. 4. Decrease in optical effect ○—○, and in viable count ●—●, after various times of heating. Samples of a suspension of *Escherichia coli* (strain 6) were heated in a water bath at 65°.

proportional manner. It may be noted, however, that suspensions treated by freezing and thawing exhibited negative optical effects, i.e. their extinction was higher in water than in NaCl solution. Moreover, bactericidal agents, in their effective concentrations, invariably abolished the o.e., while bacteriostatic substances such as antibiotics or competitive growth inhibitors (sulphadiazine) were without effect (Table 5).

Table 4. *Effect of alternate freezing and thawing on viable count and optical effect*

Suspension of *Escherichia coli* (strain 6); $E_{500}=0.20$. 18 hr. culture suspended in gelatine 0.2% (w/v), phosphate buffer 0.5% (w/v) at pH 7 was frozen by immersion into ethanol + solid CO₂ mixture. After the mass solidified it was dipped into a 25° bath. The procedure was repeated ten times. After each treatment samples were removed, washed, resuspended in distilled water and the o.e. measured.

Number of treatments	E' value	Viable cells from plate counts		Viable cells calculated from E'^* (%)
		($\times 10^7$)	(%)	
0	55	110	100	100
2	40	89	84	81
4	28	65	61	65
6	-7	22	23	21
10	-23	1	1	0

* The values in this column were calculated from the equation

$$\% \text{ of viable cells (after } n \text{ treatments)} = \frac{E'_n - E'_{10}}{E'_0 - E'_{10}} \times 100,$$

E'_0 and E'_{10} represent the o.e. before and after 10 treatments respectively, E'_n after n treatments.

Table 5. *Inhibition of the optical effect by different substances*

Organism: *Escherichia coli*; 16 hr. culture. Inhibitors in molar concentration unless otherwise stated.

	Degree of inhibition of o.e. (%)		
	100	50	0
	concentration (M)		
Cupric chloride	1×10^{-4}	2×10^{-6}	.
Mercuric chloride	2.5×10^{-6}	5×10^{-7}	.
Potassium cyanide	.	.	5×10^{-2}
Potassium ferricyanide	.	.	1×10^{-2}
Silver nitrate	1×10^{-4}	1×10^{-5}	.
Sodium azide	.	.	1×10^{-1}
Sodium meta-arsenite	.	.	1×10^{-3}
Phenyl mercuric nitrate	1×10^{-5}	5×10^{-6}	.
Sodium iodoacetate	1.7×10^{-2}	2.4×10^{-3}	.
Thiomersalate	2.4×10^{-2}	1.2×10^{-2}	.
<i>p</i> -Chloromercuribenzoate	3.5×10^{-4}	1.4×10^{-4}	.
Chloramine T	7×10^{-5}	5×10^{-5}	.
Cetylpyridinium bromide	0.001 %	0.0005 %	.
Duponol	0.05 %	0.01 %	.
Penicillin G sodium salt	.	.	2000 units
Sodium sulphadiazine	.	.	1.8×10^{-4}
Dihydrostreptomycin	.	.	1.1×10^{-4}
Phenol	1.4×10^{-2}	7.2×10^{-5}	.
2:4-Dichlorophenol	1×10^{-3}	2.5×10^{-4}	.
Formol	0.5 %	0.1 %	.
Hydrogen peroxide	0.5 %	0.1 %	.
8-Hydroxyquinoline	.	.	1×10^{-3}
8-Hydroxyquinoline + ferrous sulphate 50 mg./ml.	.	1×10^{-4}	.

DISCUSSION

Since both electrolytes and non-electrolytes cause turbidity changes in bacterial suspensions, the optical effect seems to be essentially an osmotic phenomenon. The quantitative deviations from the osmotic law might be attributed to permeability factors. It appears likely that the o.e. is due to changes in the state of swelling of the bacterial cytoplasm determined by the tonicity of the suspending medium. Plasmolysis, however, seems not to be involved since the largest increase in turbidity occurs within the 'physiological' range of the osmotic pressure (up to 0.15M-NaCl).

The reason for the absence of the o.e. in Gram-positive bacteria is not clear. The findings of Brudny (1908) and of Eisenberg (1910), that Gram-positive species cannot be plasmolysed even by very high salt concentrations, suggest that these micro-organisms are more refractory to variations in osmotic pressure than the Gram-negative bacteria. However, it is also possible that in Gram-positive bacteria the osmotic phenomenon (changes in the water content of the cytoplasm) does not manifest itself by changes in light scattering.

The existence of the optical effect (o.e.) calls for more caution in using the turbidimetric procedure for measuring cell concentration in bacterial suspensions; the failure to consider the osmotic strength of the medium may lead to up to 100 % deviations between individual estimations. On the other hand, since the o.e. seems to depend on the viability of the bacterial cell (some exceptions will be dealt with in the second part of this communication), it may be applied under carefully controlled conditions as a rapid and sensitive method for estimation of the number of viable cells.

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Turbidity Changes in Bacterial Suspensions: Kinetics and Relation to Metabolic State

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SUMMARY: The kinetics of the turbidity changes in bacterial suspensions due to variations in the osmotic pressure of the medium were investigated. The time curve of the turbidity changes followed a monomolecular course, but the velocity constant was strongly dependent on the osmotic pressure. The value of the temperature coefficient (Q_{10}) of the reaction was close to two. The rapid adjustment of the turbidity (increase or decrease) to the changes in osmotic pressure of the medium was followed by a phase of slow decline in light extinction. In this slow phase the rate of turbidity decrease was independent of the prevailing osmotic pressure, but varied with the nature of the solute used; it was markedly accelerated by KCN or HgCl_2 . The effect of HgCl_2 was annulled by thiol compounds. Certain substances affected the optical effect and the respiration in a parallel manner. The similarity between these observations and the findings of several authors with regard to turbidity changes in mitochondrial suspensions is discussed.

It has been shown in the preceding communication (Mager, Kuczynski, Schatzberg & Avi-Dor, 1956) that changes in the osmotic pressure of the medium elicited corresponding changes in the turbidity of suspensions of Gram-negative micro-organisms. This optical effect (O.E.) was tentatively attributed to changes in the state of swelling of the bacterial cytoplasm. The present study presents some further observations, which have a bearing on the nature of this phenomenon.

METHODS

The chemicals, micro-organisms and the experimental procedures employed were the same as described before (Mager *et al.* 1956). The conditions of ultra-violet irradiation are indicated in the text. For dry weight determination, the centrifuged bacterial mass was dried in a vacuum oven at 60° to constant weight. For kinetic studies, the Beckman spectrophotometer model D.U. equipped with a thermospacer for temperature control, was used.

RESULTS

Fig. 1 illustrates the kinetics of turbidity changes in suspensions of *Pasteurella tularensis* (strain S & D) elicited by different mono-monovalent electrolytes, possessing either a common cation or common anion. The increase in turbidity, due to changes in the osmotic pressure of the suspending medium, proceeded

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at a quick, but still measurable rate (phase I), which differed only slightly for the various mono-monovalent salts tested. After the maximum turbidity had been reached, the light extinction declined slowly with the time (phase II). The rate of decrease in the optical density was strongly dependent on the nature of the solute used, being especially rapid with ammonium acetate. Both the ammonium and the acetate ions are known to be highly diffusible through biological membranes (Jakobs, 1940).

The effect of the solute concentration on the rate of the turbidity changes was studied with MgCl_2 for phase I (ascending branch), and with potassium acetate for phase II (descending branch), the convenient rates of turbidity changes obtained with these solutes facilitating accurate measurements.

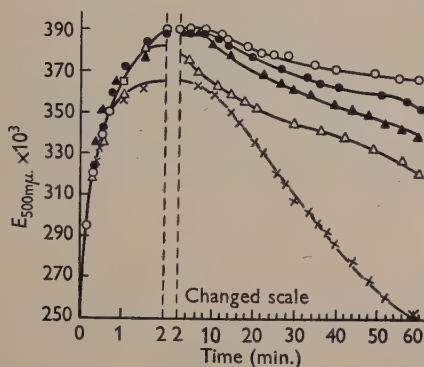


Fig. 1

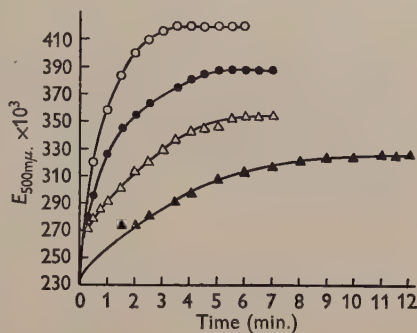


Fig. 2

Fig. 1. Kinetics of turbidity changes. Each Beckman cell contained $15 \mu\text{mole}$ solute in a total volume of 3.0 ml. ; 0.1 ml. of a suspension of *Pasteurella tularensis* (24 hr. culture) in distilled water was added at zero time. $\bigcirc-\bigcirc$, NaCl ; $\bullet-\bullet$, KCl ; $\triangle-\triangle$, NH_4Cl ; $\blacktriangle-\blacktriangle$, KNO_3 ; $\times-\times$, ammonium acetate.

Fig. 2. Effect of solute concentration on the rate of turbidity changes. Each cell contained MgCl_2 in the concentrations indicated; total volume 3.0 ml. The reaction was started by the addition of 0.1 ml. *Pasteurella tularensis* (S & D) suspension. $\blacktriangle-\blacktriangle$, 0.033 M-MgCl_2 ; $\triangle-\triangle$, 0.066 M ; $\bullet-\bullet$, 0.132 M ; $\bigcirc-\bigcirc$, 0.264 M .

It can be seen from Fig. 2 that for each solute concentration the rate of change decreased gradually, when the maximum value of the turbidity change (E'_m) was approached, while the slope of the curves became steeper with increasing MgCl_2 concentrations. Plotting of the curves on a semi-logarithmic scale yielded straight lines, thus indicating that the rate of change follows a first-order reaction course. Assuming that under otherwise constant conditions the rate of change at any instant (dE') is proportional to $E'_m - E'_t$ (where E'_t is the turbidity change at time t ; cf. first part of this communication), the following equation can be derived:

$$\frac{dE'}{dT} = k_1 (E'_m - E'_t),$$

or, in its integrated form,

$$k_1 = \frac{1}{t} \ln \frac{E'_m}{E'_m - E'_t}.$$

The experimental data for any solute concentration fit this equation well. The velocity constant k_1 , however, increases as a function of E_m (Table 1) contrary to what would be expected for a first-order reaction. Moreover, the rate of turbidity changes elicited by solutes applied in 'optically equivalent'

Table 1. *Correlation between the concentration of the solute, the maximal turbidity change (E'_m) and the velocity constant (k_1)*

Concentration of MgCl_2 ($\text{M} \times 10^{-2}$)	E'_m	$k_1 \times 10^3 \text{ sec.}^{-1}$
3.3	0.090	1.70
6.6	0.117	4.85
13.2	0.151	11.2
26.4	0.183	13.4

concentrations (i.e. producing the same o.e.) differed for mono-monovalent electrolytes on the one hand and for MgCl_2 and glucose on the other (Fig. 3). Fig. 4 shows that in contrast to the rapid phase, the rate of turbidity changes in the slow descending phase (k_2) is nearly linear and independent of the solute concentration.

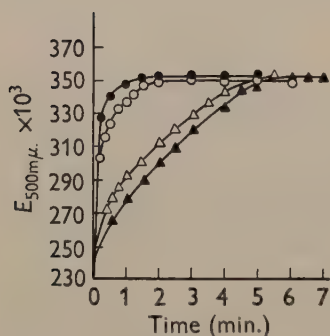


Fig. 3

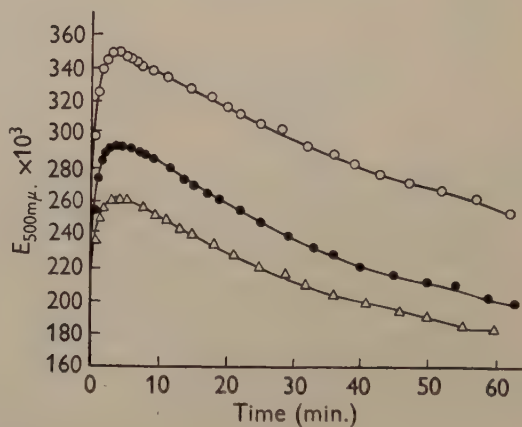


Fig. 4

Fig. 3. The velocity of turbidity changes produced by different solutes. Each cell contained the solute in the concentration indicated; total volume 3.0 ml. The reaction was started by the addition of 0.1 ml. of an aqueous suspension of *Pasteurella tularensis* (S & D). ●—●, NaCl 0.066 M; ○—○, KCl 0.066 M; ▲—▲, glucose 0.066 M; △—△, MgCl_2 0.033 M.

Fig. 4. The effect of solute concentration on rate of decrease in turbidity. Each cell contained potassium acetate in the concentrations indicated; total volume 3.0 ml. The reaction was started by the addition of 0.1 ml. of *Pasteurella tularensis* (S & D) suspension. ○—○, 0.264 M; ●—●, 0.132 M; △—△, 0.066 M.

Effect of temperature and pH value

Q_{10} for k_1 was found to be approximately 2 with either NaCl or glucose. Both E'_m and the rate constants k_1 and k_2 showed no dependence on pH value between pH 6 and 8.

Effect of HgCl₂ and KCN

It can be seen from Fig. 5 that HgCl₂ and KCN used in concentrations which were found to inhibit the respiration of *Pasteurella tularensis* (strain S & D) accelerated considerably the rate in the descending part of the turbidity change-time curves (k_2). If a suspension of this micro-organism was incubated for 1 hr. at 37° with these substances and then tested for the o.e., the influence of HgCl₂ on k_2 became even more pronounced, while that of KCN remained unchanged. The action of KCN could be reversed by washing the bacteria, while for the reversal of the effect of HgCl₂, incubation with a thiol compound was required (Fig. 6).

Incubation of suspensions of *Escherichia coli* with HgCl₂ (5×10^{-5} M, 1 hr. — 37°) inhibited the o.e. completely. When these cells were subsequently washed with thiomalate (5×10^{-2} M) and resuspended in water, the o.e. was restored to about 80 % of the original value.

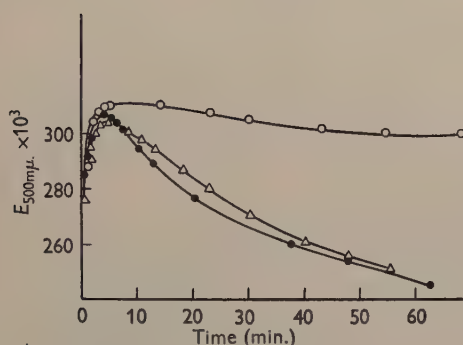


Fig. 5

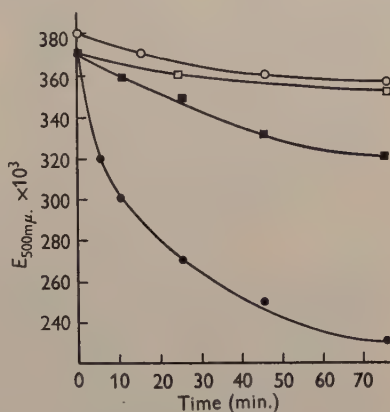


Fig. 6

Fig. 5. Effect of KCN and HgCl₂ on the velocity of turbidity changes. Each cell contained NaCl 0.132 M; total volume 3.0 ml. ○—○, control; ●—●, 10^{-2} M-KCN; △—△, 5×10^{-5} M-HgCl₂. The reaction was started by the addition of 0.1 ml. of an aqueous suspension of *Pasteurella tularensis* (S & D) 24 hr. culture.

Fig. 6. The reversal by thiomalate of the decrease of the optical effect induced by mercury chloride. A washed suspension of cells of *Pasteurella tularensis* (S & D) was incubated for 1 hr. at 37° with 5×10^{-5} M-HgCl₂ and the o.e. measured in one sample with 0.132 M-NaCl (○—○, control; ●—●, HgCl₂-treated cells). Another sample was incubated for 1 hr. with 10^{-2} M-thiomalate, washed and tested again for the o.e. □—□, control incubated with thiomalate; ■—■, HgCl₂-treated cells after incubation with thiomalate.

*Correlation between inhibition of respiration and the abolition
of the optical effect*

The above findings suggested a correlation between the inhibitory action of certain compounds on the respiration of bacteria and their effect on turbidity changes. In fact, with HgCl₂ (Fig. 7a) and with chloramine T (Fig. 7b) as well as with other respiratory inhibitors (iodoacetate, 2,4-dichlorophenol), the decrease in the o.e. started only after the respiration was strongly inhibited.

Effect of ultraviolet irradiation

It was shown previously (Mager *et al.* 1956) that certain treatments such as exposure to unfavourable pH values, alternate freezing and thawing, heating etc., produced a parallel decrease in both the viable count and the o.e. Since ultraviolet irradiation, when applied in moderate doses, sterilizes bacterial cultures without affecting their respiration (Errera, 1953), it appeared of

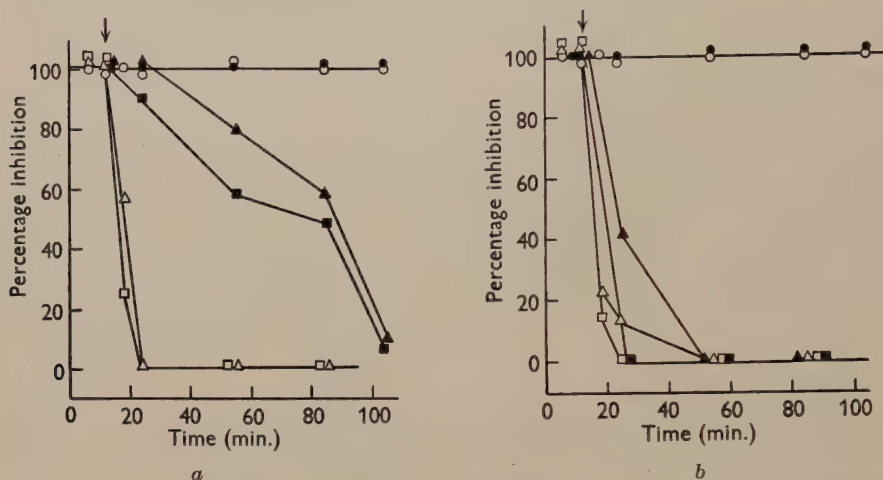


Fig. 7 *a, b.* Correlation between the action of inhibitors on the respiration and on the optical effect. Each flask contained 0.5 ml. 0.1 M-phosphate buffer (pH 7.4), 0.3 ml. 0.1 M-sodium pyruvate, and suspension of *Escherichia coli* (16 hr. culture, 5 mg. dry wt.). The final volume was made to 3.0 ml. with distilled water. The inhibitor was added from the side arm at the time indicated by the arrow. The centre-well contained 0.2 ml. 20% KOH. o.e. was tested with NaCl. The rates of O_2 uptake (unshaded marks) and the o.e. (shaded marks) were expressed in percentage of the values obtained at zero time (before the addition of the inhibitor). (*a*) \triangle — \triangle , \blacktriangle — \blacktriangle , $3.3 \times 10^{-5} \text{ M-HgCl}_2$; \square — \square , \blacksquare — \blacksquare , $6.6 \times 10^{-5} \text{ M-HgCl}_2$; \circ — \circ , \bullet — \bullet , controls. (*b*) \triangle — \triangle , \blacktriangle — \blacktriangle , $3.3 \times 10^{-4} \text{ M-chloramine T}$; \square — \square , \blacksquare — \blacksquare , $6.6 \times 10^{-4} \text{ M-chloramine T}$; \circ — \circ , \bullet — \bullet , controls.

interest to find out how this agent would influence the o.e. In Table 2 the effect of different doses of ultraviolet irradiation on viable count, respiration and the o.e. are compared. It can be seen that an exposure time sufficient for a thousandfold decrease of the viable count had no appreciable effect on the o.e. Only excessive doses of ultraviolet irradiation, which depressed respiration, diminished the o.e. too.

*Correlation between the water content of the bacterial cell and
the optical effect*

When suspensions of micro-organisms in water and in 0.5 M-NaCl were spun at 16,000 r.p.m. in a Servall angle centrifuge for 1 hr. and both the wet and dry weights of the bacterial mass were determined, significant differences were observed in the wet weight/dry weight ratio of the two respective suspensions

(Table 3). Since the intercellular space, as determined by the inulin method (Conway & Downey, 1950), was found to be equal in both suspensions, the different ratio seems to be attributable to differences in the water content of the cells. It should be pointed out that Gram-positive species behaved similarly in this respect.

Table 2. *Effect of ultraviolet irradiation on the optical effect and viable counts*

A suspension of washed *Escherichia coli*, B/r, (16 hr. culture, E_w : 0.38) was exposed in 10 ml. batches in 9 cm. diameter Petri dishes to a Claude Lumière ultraviolet lamp type S.500, 50 cm. distant, for various times as indicated. The organisms were separated by centrifugation, washed and resuspended in distilled water.

Irradiation time (min.)	Plate counts	Pyruvate $3 \times 10^{-2} M$		Glutamate $3 \times 10^{-2} M$	
		E'	Q_{O_2} ($\mu l./60$ min.)	E'	Q_{O_2} ($\mu l./60$ min.)
0	3.3×10^9	54	440	46	309
10	3.2×10^8	48	433	32	219
20	5.5×10^2	30	217	30	75
30	2.2×10^2	20	108	12	35
45	40	12	57	0	26

Table 3. *Comparison of dry weights of various bacteria sedimented from distilled water and from 0.5 M-NaCl (24 hr. culture)*

Organism	Organisms sedimented from		% Increase
	Distilled water	0.5 M-NaCl	
	Dry wt. (%)*		
<i>Alcaligenes faecalis</i>	17.8	22.6	27
<i>Escherichia coli</i> 6	18.2	22.6	24
<i>Salmonella paratyphi</i>	17.2	25.4	48
<i>Pasteurella tularensis</i> (S & D)	11.0	14.0	27
<i>Staphylococcus aureus</i>	21.0	27.6	31

* Dry weight corrected for ash.

DISCUSSION

Osmotic changes through a non-ideally semipermeable membrane (membranes of living organisms appear to belong to this type) are the resultant of two concurrent reactions: (a) transport of water in the direction opposite to the concentration gradient; (b) diffusion of the solute through the membrane in the direction of the gradient. The maximum volume changes of such a system will thus represent the net result of the two opposing processes.

The curves reflecting the turbidity changes in bacterial suspensions in relation to time (Fig. 1) can be clearly divided into two parts: a rapid phase, followed by a slow decline in light extinction. It is probable that the rapid phase corresponds to volume changes in response to an outward movement of water, whereas the slow phase is due to the readjustment of the cell volume to the gradually decreasing concentration gradient as a result of the inward

diffusion of the solute. Quite similar turbidity changes were observed in rat-heart sarcosomes (mitochondria) by Cleland (1952). The relative permeability factors calculated for NaCl or KCl from the slow phase of turbidity changes in sarcosomes are of the same order of magnitude as in *Pasteurella tularensis*. Turbidity changes in mitochondria due to variation in the state of swelling were also reported recently by other authors (Raaflaub, 1953; Price & Davies, 1954). It is assumed that the turbidity changes in mitochondria are mainly due to an alteration of the refractive index of the particles (Cleland, 1952). The correlation observed between the wet weight/dry weight ratio of micro-organisms and the tonicity of the medium affords added support for the hypothesis that changes in the state of swelling are responsible for the optical effect. The exact nature of these changes is, however, still obscure.

The course of turbidity changes in bacterial suspensions elicited by variations in the osmotic pressure obeys the law of a first-order reaction. The velocity constant (k_1) varies, however, with the solute concentration, as if the membrane permeability itself would depend on the surrounding osmotic pressure. Similar observations were reported by Lucké and co-workers in their studies on the kinetics of volume changes in eggs of *Arbacia punctulata* induced by varying osmotic pressures (McCutcheon & Lucke, 1926, 1927). They also noted the anomalously high temperature coefficient of the osmotic process. Later Lucké, Hartline & McCutcheon (1931) showed that with an improved equation, which takes account of the concomitant changes in the surface area of the cell, the dependence of the rate of swelling on the osmotic pressure can be explained.

As previously reported (Mager *et al.* 1956), the o.e. is exhibited only by living micro-organisms. The reversible abolition of the o.e. by HgCl_2 and the correlation between the effect of certain agents (bactericidal substances, ultra-violet irradiation) on respiration and turbidity changes, indicate that the more direct reason for the disappearance of the o.e. might probably be the decrease of the rate of respiration below a certain critical level. A correlation between the state of swelling and respiration in tissues (Elliot, 1946; Stern, Eggleston, Hems & Krebs, 1949) and in mitochondria (Macfarlane & Spencer, 1953; Bartley & Davies, 1954) has been observed by many authors. The analogous findings in bacteria presented in this paper suggest that a similar mechanism may be involved in regulating the metabolic state of the bacterial cell.

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A Nuclear Gene Suppressor of a Cytoplasmically Inherited Character in *Neurospora crassa*

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SUMMARY: The cytoplasmically inherited trait in *poky*, *Neurospora crassa*, is recognized by certain defects in the cytochrome system accompanied by a low growth rate. In the presence of the gene, *f*, the growth rate of a *poky* strain becomes nearly normal but the defective cytochrome system remains unchanged, as if the defects were being compensated for by an increase in activity of some other enzyme system. When the *f* allele of this gene is replaced by its normal counterpart the *poky* character is again fully expressed. The allele, *f*, is apparently without effect on another cytoplasmically inherited character, *mi 3*, similar in its properties to *poky*. Nor do the nuclear gene mutants, C115 and C117, also similar in phenotype, appear to respond to *f*. A nuclear gene suppressor of the mutant, C115, restores to normal not only the growth rate but also the cytochrome system of this mutant. No effect of this suppressor on *poky*, *mi 3* and C117 has been detected.

A partial suppressor of the *poky* character (Mitchell & Mitchell, 1952*a*) was found in a *poky* isolate of *Neurospora crassa*. This isolate, after being maintained for several years by vegetative transfer, was observed to have changed so that, with respect to its growth rate, it was more like wild *N. crassa* than like *poky*, but the cytochrome content of the mycelium (Tissieres & Mitchell, 1954) remained much the same as that of typical *poky*. Upon examination it appeared that a gene mutation was responsible for this change. The genetic tests which indicate this and tests to determine the effects of this gene on three other cytochrome-defective strains (Mitchell, Mitchell & Tissieres, 1953) are reported here. The reactions of these strains with a suppressor of one of the nuclear gene cytochrome mutants are also reported.

The suppressor of poky in poky cytoplasm

The fast-growing isolate of *poky*, *po*-1720-2*a*, was crossed to wild-type conidia, and asci were dissected on agar plates. All spore pairs from 14 asci produced mycelia which were phenotypically *poky* when examined about 20 hr. after heat treatment. The isolates from six of these asci, each from a different perithecium, were transferred to slopes and after 3 or 4 days it was obvious that two of the four isolates from each of five of the asci were growing more rapidly than *poky*. Isolates from the sixth ascus all remained typically *poky*. This suggests that *po*-1720-2*a* was heterocaryotic with respect to a Mendelian factor which permits more rapid growth.

Reciprocal crosses were then made between a slow and a fast isolate (*po* + -3564-4*A* and *po f*-3564-3*a*) from the same ascus. From each cross

20 asci were dissected, the germinated spore pairs of which were transferred to slopes. The two crosses gave the same result, namely, that in each tetrad 1:1 segregation of fast and slow *poky* had occurred. From this it appears that the gene *f* has the same effect regardless of whether it is contributed by the conidial (paternal) parent or is present in the protoperithecial (maternal) parent. Also, it is again indicated, as it was by the *po*-1720-2a cross, that *poky* can be recovered unchanged after having harboured the *f* form of the gene.

Isolates from the 40 asci were tested for mating type reaction, but no linkage of *f* to the mating type locus was indicated. Other linkage tests have not been performed.

Ten tetrads from *po* + × *po f* were cultured in 125 ml. flasks of minimal medium and examined with respect to dry weight and cytochrome content of the mycelium. The cytochrome bands which could be detected with a hand spectroscope differed very little in moist mycelium from the fast and slow isolates. In the *f* isolates the *c* band could usually be seen to be slightly weaker, and the *b* band slightly stronger than in the + isolates. The *a* + *a*₃ band could not be seen in either. The difference may, perhaps, be due to the greater physiological age of the fast isolates, since it is known that typical *poky* changes in this direction with age (Haskins, Tissieres, Mitchell & Mitchell, 1953). Dry weights (in mg.) of mycelium obtained from the 4-day cultures may be summarized as follows:

	Highest	Lowest	Average
<i>f</i> cultures	66	48	58
+ cultures	25	7	15

Since the dry weights from the *f* cultures approach very nearly that of standard wild type it appears that the growth rate of *poky* can be restored almost to normal without any marked effect on the cytochrome content becoming detectable. Examination of the enzyme system or systems responsible for this restoration is in progress.

The suppressor of poky in wild-type cytoplasm

From the cross, wild-3177-4A × *po f*-3564-3a, 26 asci were dissected, and the spore pairs allowed to germinate on minimal plates. All isolates were phenotypically wild on the plates and no significant differences in growth rate were detected among those from 10 of the asci transferred to slopes. Dry weights (in mg.) from 4-day flask cultures of these 40 isolates were as follows: highest, 83; lowest, 64; average, 71.

From one tetrad (3585) isolates 1 and 2 were crossed to *po f*-3564-3a protoperithecia and 3 and 4 were crossed to protoperithecia of *po* + -3564-4A. Asci were dissected and the spore pairs of two from each cross were cultivated on slopes. The crosses of isolate 3585-2 and -4 gave 2 *po f* and 2 *po* + pairs per ascus; the cross of isolate 1 gave only *po f* pairs and that of isolate 3 gave only *po* +. Hence the constitution of ascus 3585 was *f* + + *f*. The four 3585

isolates were cultivated in flasks so that their mycelia could be examined for cytochrome bands. The bands seen in the two isolates shown to carry *f* did not differ from those seen in the + isolates or in standard wild type.

The suppressor of poky in mi 3 cytoplasm

Strains showing the cytoplasmically inherited character, *mi 3*, resemble *poky* in having an excess of cytochrome *c* and no *a + a₃*, but they appear to be normal with respect to *b* and have *a₁*. They grow two or three times faster than *poky*.

An isolate of *mi 3* was crossed as protoperithecial parent to *f* in wild-type cytoplasm (*mi 3*-2543-1a × *f*-3585-1A). All spore pairs from 26 asci were phenotypically like *mi 3* on the minimal plates and those from 10 asci transferred to slopes remained so throughout their growth. Dry weights (in mg.) from 4-day flask cultures of these 40 isolates were as follows: highest, 38; lowest, 11; average, 28.

From ascus 3754 isolates 1 and 4 were crossed to protoperithecia of *po*-3627-1A and 2 and 3, to *po*-3627-2a, in order to see which isolates carried *f*. Two tetrads from each cross were examined. Those from the crosses of isolates 1 and 4 contained only *po* + segregants, whereas those from the crosses of isolates 2 and 3 contained two *po f* and two *po* + spore pairs. Ascus 3754 was, therefore, of the constitution + *ff* +. Mycelia from flask cultures of these four isolates were found not to differ, with respect to cytochrome bands, from typical *mi 3*.

It appears then, that *f* can be present in *mi 3* cytoplasm without producing any detectable effect on the growth rate or cytochrome content.

Combinations of the nuclear gene mutants with the suppressor of poky

The two gene mutants, C115 and C117, grow slowly, like *poky* and *mi 3*, and show an abnormal content of cytochromes. C115 resembles *poky* in cytochrome content, except that the excess of *c* and the deficiency of *b* are less pronounced. C117 contains cytochromes *b* and *e* but is deficient in *c* and *a + a₃*.

Spore pairs of five asci from *f*-3585-4a × C115A were cultivated on slopes. The C115 isolates were essentially alike in growth rate. Five of these were crossed to protoperithecia of *po*-3627-3a. By this time it had been observed that, on agar plates, mycelia from *po f* spores could be distinguished from those of *po* + spores by the more rapid growth of the former at 35°. When random spores from the above five crosses were examined in this way *po f* spores were recovered from two of the crosses but were not found from the other three. Dry weights of mycelium from flask cultures of the five C115 isolates did not differ significantly from each other or from those obtained from other C115 isolates, nor were differences in cytochrome bands observed. Hence it is concluded that *f* does not influence the growth rate and cytochrome content of C115.

When 26 tetrads from the cross, *f*-3585-4a × C117A, were examined on plates the spore pairs showed 1:1 segregation of mutant and wild with no indication

of suppression of the mutant. Spore pairs from 10 of these asci were cultivated on slopes of complete medium and observed during their growth. The behaviour of the mutant isolates was like that of typical C117.

In order to show that C117 *f* recombinants were actually obtained, tetrads from the cross, *po f*-3627-3a \times C117A, were grown on slopes. As previously observed with the cross of *poky* \times C117, many of the isolates carrying C117 died after producing short germ tubes or a few strands of mycelium. However, in six of the ten tetrads observed, one of the two *poky* isolates not carrying C117 also did not carry *f*. In one tetrad there were two *po* ++ isolates. From this it is concluded that there is no linkage which prevents frequent recombination of *f* and C117. It seems safe, then, to conclude that *f* does not change the phenotype of C117.

A suppressor of C115

An apparently reverted isolate of C115 has been found to carry a suppressor (designated as *s*) of this mutant. This 'reverted' strain was crossed to wild-type protoperithecia and asci were examined. On the minimal plates there was clearly 1:1 segregation of mutant and wild in each tetrad, but when the isolates were grown on slopes some of those which had been phenotypically mutant on the plate grew much more rapidly than C115. A tetrad (2522) was selected of which isolates 1 and 2 had been phenotypically wild on the plate. Isolate 3 had been mutant on the plate but was fast-growing in slope culture, and 4 had been, and remained, mutant. Isolates 1 and 2 were crossed to C115 and 3 was crossed to wild. The cross of 1 gave, in tetrads, 1:1 segregation of wild and typical C115, whereas the crosses of 2 and 3 both gave tetrads which were like those from the cross of the 'reverted' strain. The constitution of tetrad 2522 was, therefore, ++ + *s* C115 *s* C115 +. When isolates 1, 2 and 3 were cultivated in flasks no differences were found in growth, cytochrome bands and response to yeast extract, which inhibits growth of C115. With respect to the properties by which the mutant, C115, is characterized the suppressed mutant differs from wild type only in initial growth rate (from ascospores).

Isolates from 16 asci from *s*-2522-2 \times C117 behaved, on plates and slopes, like those from crosses of C117 to wild without *s*. All C117 isolates except one made fully grown slope cultures and were tested in flasks. With respect to dry weight and cytochrome bands these 31 isolates did not differ from typical C117. There is, however, no evidence that *s* actually combined with C117. This cannot be demonstrated by having C115 present in the cross of C117 to *s* since C115 *s* \times C117 is, like C115 + \times C117, completely sterile.

Protoperithecia of *mi* 3 were crossed to conidia of *s*-2522-2. Spore pair isolates from 10 asci showed no differences from typical *mi* 3 on plates, slopes, and in flask cultures. From this it appears that *s* does not affect the *mi* 3 phenotype. There does, however, appear to be an effect of *mi* 3 on suppression of C115 by *s*. This effect was observed when C115 *s*-2522-3 was crossed to *mi* 3 protoperithecia in order to show that *s* was indeed present in *mi* 3. If *s* does not affect *mi* 3 and if C115 is as fully suppressed in *mi* 3 cytoplasm as it is in wild, then the progeny of this cross should exhibit only two phenotypes in flask

culture, *mi 3* and C115 in *mi 3*, in tetrad ratios of 4:0, 3:1 and 2:2. (C115 in *mi 3* grows very slowly but is like C115 with respect to cytochrome bands, except that the *c* band is much stronger.) Actually there were three phenotypes, the third being intermediate in growth rate. Segregations were such as to suggest that the third type was C115*s* in *mi 3*, since in each of ten tetrads there were two isolates like *mi 3*. The other two were both like C115 in *mi 3*, both intermediate, or one of each of these types. From a tetrad containing one C115 in *mi 3* and one intermediate type, the two *mi 3* isolates were crossed to the intermediate one. One of these crosses gave only *mi 3* and 'intermediate', whereas the other gave all three types as in the parent cross. This result is consistent with the constitution of the tetrad being as follows: $+s$ in *mi 3*; $++$ in *mi 3*; C115*s* in *mi 3*; C115 $+$ in *mi 3*. Tests in flask culture of tetrads from *s* in *mi 3* \times C115*s* in *mi 3* confirmed the previous observation that *s* does not affect *mi 3*, since there were two typically *mi 3* isolates in each tetrad. The 'intermediate' isolates were much like C115 in *mi 3* with respect to cytochrome content.

Tetrads from *poky* \times C115*s* were also examined and 20 of these which were cultivated on slopes were found to contain no isolates which grew faster than typical *poky* strains. Five tetrads, each containing one isolate classified as C115 $+$ in *po* (this combination is also very slow-growing, shows a great excess of cytochrome *c* but other cytochrome bands have not been seen) were tested in flasks. Again, two isolates per tetrad did not differ from *poky*, either in growth or in cytochrome content. The previous classification of C115 $+$ in *po* isolates was confirmed. The fourth isolate in each case, presumably C115*s* in *po*, grew more slowly than *poky* and was more like C115 $+$ in *po* with respect to cytochrome bands, although the excess of *c* was less pronounced. Thus it appears that *poky*, like *mi 3*, is not affected by *s* and that *poky* also interferes with the suppression of C115 by *s*.

DISCUSSION

The observation that *f*, the partial suppressor of *poky*, does not seem to affect the cytochrome content of any strain tested suggests that *f* increases the growth rate of *poky* by enhancing the activity of a compensating enzyme system. Should this be true, then the failure of *mi 3*, C115 and C117 to respond to *f* may mean either that this system cannot compensate for those which are defective in these three strains, or that, because of the nature of the defects, the compensating system cannot be enhanced.

That *f* fails to repair *mi 3* is of some interest from the standpoint of inheritance. Although the two cytoplasmically inherited characters, *poky* and *mi 3* are quite similar, they have been found to differ consistently in growth rate, cytochrome content and in activities of enzymes studied *in vitro*. The difference in their reactions with *f* serves to distinguish them further.

Since C115 with its suppressor appears to become normal in cytochrome content as well as in growth rate, the action of *s* in restoring C115 may be through the cytochrome system itself. If so, the abnormalities in the cyto-

chrome system by which *poky* and *mi 3* are characterized must be different from those which give rise to the C115 phenotype, since *poky* and *mi 3* are not repaired by *s*. This is consistent with the observations that *poky* and *mi 3* interfere with suppression of C115 and also that an additive effect is obtained when C115 is installed in either *poky* or *mi 3* cytoplasm.

The lag observed in the growth of both *po f* and C115 *s* from ascospores is like that found earlier in connexion with a suppressor of pyrimidine-requiring mutants (Mitchell & Mitchell, 1952*b*). Suppression by means of an adaptive mechanism is suggested. It is of interest that in the case of the defect inherited as a Mendelian character (C115), it is the defective system itself which appears to adapt, whereas in *poky* the defective cytoplasm appears to remain unchanged and the adaptation to take place in a compensating system.

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An Immunological Study of the Constitutive and the Penicillin-induced Penicillinases of *Bacillus cereus*, Based on Specific Enzyme Neutralization by Antibody

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SUMMARY: Exocellular penicillinases from strains 569 and 5/B of *Bacillus cereus* gave almost identical activity-neutralization curves when titrated with antisera prepared against the enzyme from either strain; but were easily distinguishable by differences in their neutralization reaction with an anti-569 penicillinase serum previously partially absorbed with either the 5/B or the 569 enzyme. By contrast, no differences in neutralization reaction were detected between basal and induced penicillinase from strain 569 and the constitutive penicillinase from strain 569/H (a mutant derived from strain 569), when treated with samples of the anti-569 penicillinase serum, previously partially absorbed with each penicillinase preparation separately.

Strain 569 was not found to produce any substance in significant quantities capable of combining with anti-penicillinase neutralizing antibodies other than penicillinase itself.

Although it has been known for many years that enzymically active proteins may evoke the production of precipitating and neutralizing antibodies in animals, relatively little systematic work has been done on the neutralization reaction. Toxin neutralization by specific antitoxin has of course been investigated in detail, but, except in those cases where the toxin is itself enzymically active and has a well-defined substrate, such studies suffer from the disadvantages of cumbrous and inaccurate *in vivo* titration of biological activity. In those instances where an enzyme stimulates the formation of antibodies which will neutralize—to a greater or lesser extent—enzymic activity, the antigen-antibody reaction can be studied quantitatively at relatively low concentrations of the reagents from an aspect which is distinct from the precipitation reaction and can thus be expected to shed new light on the character of the specific combination. Some of the possibilities offered by such studies of enzyme-antibody reactions have been outlined in a stimulating article by Cinader (1953); and the subject has been reviewed by Sevag (1951).

Quite apart from its general immunological interest, the pioneer work of Cohn & Torriani (1952, 1953) on the properties of induced β -galactosidase and the related protein Pz in *Escherichia coli* has shown clearly the advantages to be gained by the application of immunological methods to the study of induced enzyme synthesis in micro-organisms. Housewright & Henry (1947) showed that penicillinase from *Bacillus cereus*, strain 569 can evoke the production of penicillinase-inhibiting antibodies in rabbits. The present work was begun as an immunological analysis of penicillinase induction, analogous to that carried out by Cohn & Torriani on β -galactosidase formation in *Escherichia*

coli, and has been extended to include a detailed immunological comparison of the induced, basal, and constitutive exocellular penicillinases formed by *Bacillus cereus* strains with or without treatment by penicillin (see Kogut, Pollock & Tridgell, 1956).

METHODS

Enzyme preparations for immunization. (a) Induced penicillinase from *Bacillus cereus*, strain 569 grown in a peptone medium, and purified (Pollock & Torriani, 1953) to an extent which subsequent work (Kogut *et al.* 1956) showed was about 50 % pure. Subsequently referred to as induced 569 penicillinase.

(b) Constitutive penicillinase from *Bacillus cereus*, strain 5/B—a strain physiologically distinct from strain 569—being a constitutive penicillinase mutant derived from the non-inducible *B. cereus*, strain 5 (Sneath, 1955): used at a stage in isolation which further work (Pollock, Torriani & Tridgell, 1956) showed to have been 60 % pure. Subsequently referred to as 5/B penicillinase.

Enzyme preparation for in vitro reactions with antibodies. (a) Induced 569 penicillinase: apparently pure, as judged by electrophoretic and ultracentrifugal analysis (Kogut *et al.* 1956).

(b) Basal penicillinase from *Bacillus cereus*, strain 569: formed without induction by penicillin (impure preparation: see Kogut *et al.* 1956); subsequently referred to as basal 569 penicillinase.

(c) Constitutive penicillinase from *Bacillus cereus*, strain 569/H (constitutive mutant from strain 569): apparently pure, judged by electrophoresis and ultracentrifugal analysis (Kogut *et al.* 1956); subsequently referred to as 569/H penicillinase.

(d) 5/B penicillinase: crystalline preparation (Pollock *et al.* 1956).

Immunization. (a) Induced 569 penicillinase. 4 mg. of the enzyme preparation in a volume of 2.0 ml. were injected into the subscapular region of each of three rabbits (359, 360 and 362) after homogenizing with Freund's adjuvant (Freund, Thomson, Hough, Sommer & Pisani, 1948). Four weeks later each rabbit received five further intravenous injections on alternate days of about 0.5 mg. of enzyme preparation adsorbed on aluminium hydroxide. This 'alum precipitated' enzyme was prepared by adding 7.5 ml. of the enzyme solution in 0.01 M-phosphate (pH 7.0) to 0.25 ml. of 1 % ammonium alum. The aluminium hydroxide, with adsorbed enzyme, was allowed to flocculate overnight and resuspended by mixing before injection. The rabbits were bled white 10 days after the last injection and the sera separated. The sera are subsequently referred to as anti-569. The serum from each rabbit was kept separate and distinguished by the animal's number added in brackets, e.g. anti-569 (362).

(b) 5/B penicillinase. Three rabbits (1, 2 and 3) each received six intravenous injections of about 0.5 mg. of alum-precipitated enzyme per injection over the course of 3 weeks, and were bled white 8 days after the last injection, and the sera separated. The sera are subsequently referred to as anti-5/B.

Further treatment of antiserum. The crude γ -globulin fraction of rabbit 362 antiserum (which had the highest anti-penicillinase titres and on which

most of the work was done) was precipitated with ethanol by a technique based on that described by Nichol & Deutsch (1948) as follows. 50 ml. of serum were diluted to 150 ml. with 0.85 % saline, adjusted to pH 7.6 by addition of N-HCl and cooled in a freezing bath until ice crystals began to form. 50 ml. ethanol, previously cooled to -20° , were added slowly, with constant stirring, and the precipitate allowed to settle ($1\frac{1}{2}$ hr.), centrifuged off and dissolved in saline containing 0.01 M-phosphate pH 7.0 (vol. 20 ml.). The solution was freeze-dried in ampoules in 2.0 ml. lots (corresponding to 5.0 ml. of the original serum) and dissolved in 5.0 ml. saline before use so that antibody concentration was the same as that in the original serum and antibody titres could be compared directly with those in untreated whole sera. The overall loss of anti-penicillinase activity by this precipitation method was found to be negligible. The other antisera were freeze-dried and sealed in ampoules, without further treatment.

Enzyme neutralization tests. These correspond to constant antigen titrations. They were done at very low concentrations of antigen (from 0.03 to 0.2 μ g. purified enzyme/ml.) and antibody in the presence of 1 % (w/v) gelatin by mixing a series of 1.0 ml. samples of the enzyme solution with 0.5 ml. of saline containing varying concentrations of antibody. The mixture was allowed to stand 30 min. at room temperature, and the enzyme activity assayed, on 1.0 ml. samples, manometrically by the method of Henry & Housewright (1947). The error of this assay technique is not more than 5 %. Experiments on antibody from rabbit 362 showed that the neutralization reaction between enzyme and antigen was complete within 10 min. at room temperature. In antigen excess the amount of enzyme activity neutralized (whatever the concentration of enzyme) is directly proportional to the quantity of antibody added, so that it is permissible to define an anti-penicillinase neutralization unit (N.U.) as being that quantity capable of neutralizing 1 unit of enzyme (defined by Pollock & Torriani, 1953) in a mixture where enzyme is in excess (i.e. not all in combination with antibody). In practice, the neutralization titre of any given antibody solution can be calculated directly from the slope of the linear portion of the neutralization curve (see later in text). Repeat estimations of neutralization titre against one particular enzyme never differed by more than 20 %. Normal rabbit serum, diluted 1/2, produced no detectable inhibition of 100 units induced 569 enzyme/ml.

Antibody absorption test. This corresponds to a constant antibody titration. Varying amounts (usually between 10 and 20 μ g.) of enzyme were added to a constant amount of antibody (usually corresponding to between 0.06 and 2.5 ml. of undiluted serum) in saline containing 0.01 M-phosphate (pH 7.0) in a final volume of 3.0 ml. At this relatively high enzyme concentration no gelatin is necessary in order to prevent inactivation. The mixture was left at 35° overnight, and the precipitate spun off, washed when necessary, and resuspended in the same volume of buffered saline. The supernatant fluid was assayed for enzyme activity after suitable dilution in 1 % aqueous gelatin and its residual enzyme-neutralization titre estimated as described above. The enzyme activity in the evenly resuspended precipitate was similarly assayed.

RESULTS

Enzyme neutralization

Fig. 1 illustrates a typical neutralization curve obtained when increasing amounts of anti-569 (362) antibody were added to a given quantity of 569 induced penicillinase. The following points should be emphasized:

(1) After mixture with antibody the enzymic hydrolysis of penicillin proceeds at a decreased but strictly linear rate in the usual way until at least 95 % of the substrate is destroyed; and five-fold variation in initial penicillin concentration (10^{-2} M to 2×10^{-3} M) caused no difference in the rate of hydrolysis by antibody-inhibited enzyme. It can therefore be concluded that, once

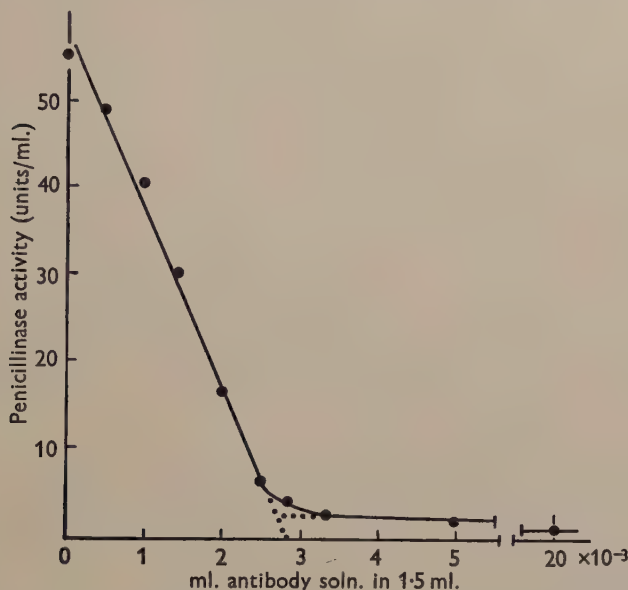


Fig. 1. Neutralization curve of purified induced 569 penicillinase with anti-569 penicillinase γ -globulin (362) preparation.

combination between enzyme and antibody has occurred, it cannot easily be reversed by addition of substrate. The effect of adding antibody to enzyme-substrate mixtures (i.e. during the course of penicillin hydrolysis) has not been studied here, although Housewright & Henry (1947) found that maximum inhibition of enzyme activity was attained within 60 min. of antiserum addition.

(2) The curve is approximately linear over 95 % of its course—i.e. the amount of enzyme activity neutralized is proportional to the quantity of antibody added. From the slope the anti-569 neutralization titre of the antibody preparation can be calculated.

(3) The neutralization titre is not affected significantly either by variations in the concentration of enzyme employed or the presence of other substances. It was found that the antibody had quantitatively the same neutralizing action

against pure enzyme preparation as against enzyme in untreated culture supernatant fluid. Even the presence of intact cells or extracts from crushed cells (from an uninduced culture of strain 569) at a concentration of 1 mg. dry weight/ml. was not found to affect the neutralization reaction significantly.

(4) The small amount of residual activity not neutralizable even by gross excess of antibody is apparently due to the activity of the enzyme-antibody complex itself. This was shown in precipitation tests by resuspending the precipitate and measuring its enzymic activity in the usual way (see Table 1 and later in text). As long as antibody was in excess no significant enzyme activity was detectable in the supernatant fluid after removal of the enzyme + antibody precipitate by centrifugation. All the residual activity, under such conditions, remained in the precipitate. The true equivalence point—(i.e. antigen-combining titre), where enzyme and antibody are in combination with no appreciable free enzyme or antibody present—is thus probably best indicated by the point of intersection of the linear portion of the neutralization curve with the extrapolation of a line joining the points obtained in the region of antibody excess. In the case illustrated in Fig. 1, the activity of enzyme-antibody complex was very low and the enzyme-combining titre of antibody did not differ much from the enzyme-neutralizing titre. But in other cases (see Fig. 2*a*, *b*, and later) there may be a big difference. It was confirmed by enzyme/antibody precipitation tests (in excess antibody) that, in the case of anti-569 (362) antibody the quantity of neutralizing antibody removed by precipitation with enzyme did not differ by more than 20 % from that expected from the antigen-combining titre calculated from direct enzyme-neutralization curves.

Homologous and heterologous neutralization

Fig. 2 illustrates the four pairs of neutralization curves obtained when two anti-569 (Fig. 2*a* (i) and (ii)) and two anti-5/B (Fig. 2*b* (i) and (ii)) sera were titrated against both induced 569 and 5/B penicillinases. The salient points may be summarized:

(1) Although the four antisera differed from one another considerably in their neutralization titres, there was no marked difference between the two enzymes in the extent to which they were neutralized by any of the antisera.

(2) The two anti-5/B sera had lower neutralizing titres and gave much higher residual activities than the two anti-569 sera. Since the anti-5/B and anti-569 sera were not prepared by the same immunization technique it is not possible to decide whether this was due to differences in the immunizing antigen or in the immunizing technique. However, the two anti-5/B sera themselves differed from one another both in neutralization titre and residual activity in antibody excess. Calculations show, however, that the equivalence point (i.e. the antigen-combining titre) is almost exactly the same for the two sera. They probably differed, therefore, *only* in the activity of the enzyme-antibody complex. Since in this case the immunization techniques and immunizing antigens were identical, the variation must lie in the antibody-forming systems of the two rabbits such that the induced antibody molecules apparently differed in their ability to neutralize the enzyme activity when combined.

Precipitation tests

Enzyme-antibody precipitates. Table 1 shows the proportions of the original enzyme activity remaining in the resuspended precipitate formed after addition of antibody. The values correspond approximately to the proportion of residual activity in antibody excess found in the direct neutralization test,

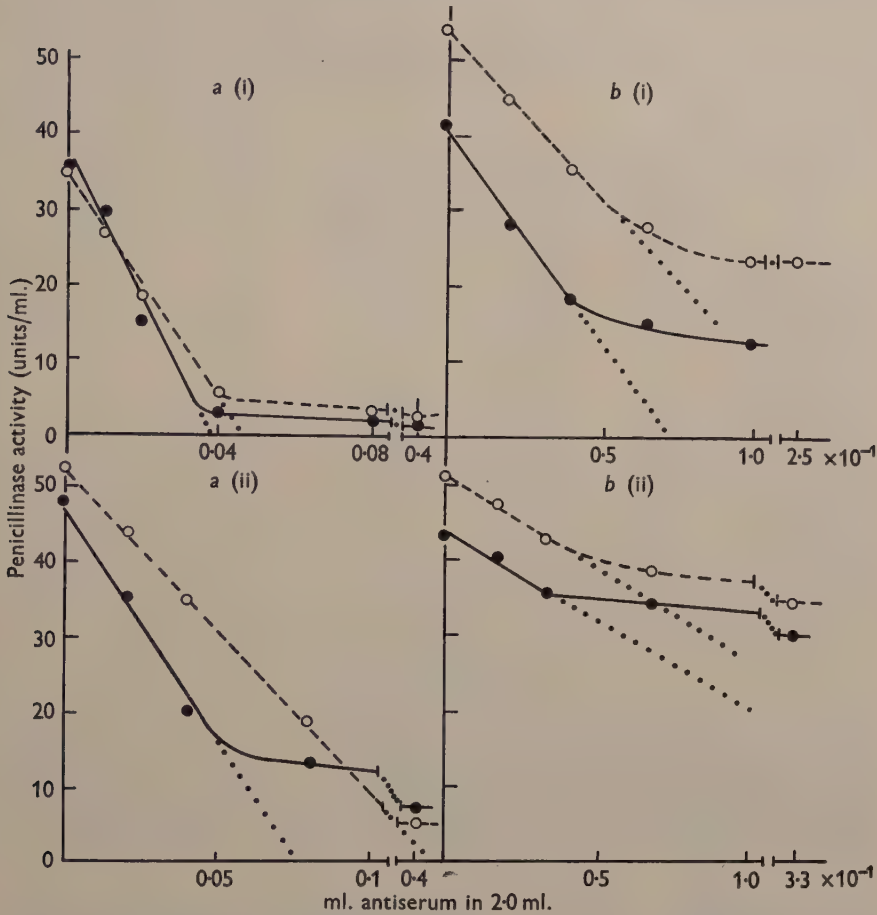


Fig. 2. Neutralization of penicillinase activity by four antisera (unabsorbed) prepared against: (a) induced 569 penicillinase in (i) rabbit 362, (ii) rabbit 360, and (b) 5/B penicillinase in (i) rabbit 1, (ii) rabbit 2; and titrated against both induced 569 (—●—●—) and 5/B (---○---○---) penicillinases.

and there is clearly a marked difference between anti-5/B and anti-569 antisera in the extent to which enzyme activity is neutralized by combination with antibody. The enzyme+antibody precipitate (washed twice with saline) formed in excess enzyme appeared to be slightly more active than when formed in excess antibody. With anti-569 (362) antibodies, the enzyme+antibody precipitate appeared to be almost completely insoluble and the complex to be

stable (at least in the absence of substrate). Repeated washing and resuspension of the precipitate in saline did not cause any significant decrease in its activity, or appearance of activity in the washings (Table 2).

Table 1. *Enzyme activities of penicillinase/anti-penicillinase precipitates*

Varying quantities of enzyme and antiserum were mixed in a final vol. of 3.0 ml. saline containing 0.01 M-phosphate (pH 7.0) and, after incubation at 35° for 16 hr., the precipitate was centrifuged down, washed in buffered saline, resuspended and assayed for enzymic activity. The supernatant fluid was assayed for enzyme activity and titrated for neutralizing ability against the enzyme used for precipitation.

Expt.	Anti-penicillinase immune serum		Precipitating penicillinase		Neutralizing antibody titre remaining in supernatant (%)	Penicillinase activity remaining in	
	Type	Vol used (ml.)	Type	Quantity used (units)		Super-natant (%)	Precipitate (%)
I	Anti-569 (362)	0.25	5/B	3600	15.6	0.7	3.7
II	Anti-569 (362)	0.25	5/B	3500	30	<0.5	1.9
	Anti-569 (362)	0.25	Induced 569	5100	38	<0.5	1.6
	Anti-569 (362)	0.25	Basal 569	5100	34	<0.5	1.7
			(impure)				
	Anti-569 (362)	0.25	569/H	5100	35	<0.5	1.7
III	Anti-569 (362)	0.20	5/B	3500	13	<0.5	2.2
	Anti-569 (362)	0.10	5/B	2500	<1	0.8	2.5
	Anti-569 (362)	0.08	5/B	2500	<1	10.5	4.7
	Anti-569 (362)	0.06	5/B	2500	<1	34	3.6
	Anti-569 (362)	0.10	5/B	5000	<1	45	4.6
IV	Anti-5/B (1)	2.35	5/B	3600	<1	0.4	54
	Anti-5/B (1)	2.35	5/B	5350	<1	2.9	59

Table 2. *Stability of penicillinase/anti-penicillinase precipitate on repeated washing*

21,300 units of induced 569 penicillinase were mixed with 0.75 ml. of anti-569 (362) γ -globulin (i.e. antibody excess) in 9.0 ml. of buffered saline. After incubation at 35° for 16 hr. the precipitate was centrifuged down and resuspended in the same volume of buffered saline. After leaving for at least 24 hr. at +2° the enzymic activity was assayed in duplicate, the precipitate centrifuged down again and resuspended, at the same concentration, in buffered saline, while the supernatant fluid from the centrifugation was also assayed for activity. This procedure was repeated five times.

No. of resuspensions of precipitate in buffer saline	Penicillinase activity of	
	Resuspended precipitate	Washings
	(Units/ml.)	
1	46.5	<1.0
2	51.0	<1.0
3	51.5	<1.0
4 Immediately after resuspension	48.5	<1.0
After standing 24 hr.	49.2	
5	51.2	1.0

Complete absorption of antibodies. By adding enzyme to antibody to the point when free enzyme began to appear in the supernatant fluid after centrifuging off the precipitate (= equivalence point), it was possible to remove all, or very nearly all, the neutralizing antibodies to both types of enzyme with equivalent amounts of either 5/B or induced 569 penicillinase (see Table 3).

Table 3. *Removal of neutralizing antibodies from anti-penicillinase immune serum by absorption with homologous and heterologous penicillinases*

The antibody and enzyme solutions were mixed in a final vol. of 3.0 ml. saline containing 0.01 M-phosphate (pH 7.0) and incubated for 16 hr. at 35°, after which the precipitate was centrifuged down and the supernatant fluid assayed for penicillinase activity and penicillinase-neutralizing ability.

Amount of anti-569 penicillinase serum (ml.)	Absorbing penicillinase		Enzyme activity remaining in supernatant after absorption (%)	Neutralizing anti- body titre remaining (after absorption) against	
	Type	Quantity (units)		Induced 569	5/B
				%	
0.25	Induced 569	8700	0.4	0.9	1.4
0.25	5/B	5500	4.0	2.0	< 0.5

Table 4. *Constant antibody titration for determining the equivalence point of combination between 5/B penicillinase and anti-569 antibody in the precipitation reaction*

Varying quantities of crystalline 5/B penicillinase were mixed with 0.06 ml. of anti-569 (362) undiluted γ -globulin solution in physiological saline containing 0.01 M-phosphate (pH 7.0) in a final volume of 3.0 ml. and incubated for 16 hr. at 35°. The precipitate was centrifuged off and the enzyme activity remaining in the supernatant fluid was assayed. 'Equivalence' point of enzyme/antibody reaction expressed as amount of 5/B enzyme combined with 1.0 ml. undiluted antibody solution: calculated from table below: 24,970 units; calculated from neutralization curve (not shown): 26,800 units.

Amount of 5/B penicillinase added (units)	Total amount of penicillinase found in supernatant after removal of precipitate (units)	Total amount of penicillinase precipitated (units)
1340	< 6	1334
1600	46	1554
2000	395	1605
2400	1040	1360
2800	1310	1490
3200	1720	1480

Mean
value:
1498

When larger amounts of enzyme were added, all the excess enzyme activity appeared in the supernatant fluid; Table 4 shows that the total amount of enzyme precipitated by a given amount of antibody did not vary significantly, after the equivalence point was reached, even in the regions of marked antigen excess.

Up to this point there appeared to be no clear evidence of any immunological differences between the induced 569 and 5/B penicillinases. Indeed, it might

well have been argued that the similarity of the neutralization slopes and the results of the absorption tests were good evidence in favour of the two enzymes being identical. However, it was noticed on repeated tests that anti-569 (362) antibody gave consistently slightly higher neutralization titres against the induced 569 penicillinase than against the 5/B enzyme, the mean difference being 35 %. Further work, restricted entirely to the higher titre anti-569 (362) antiserum, on the effect of partial absorption of antibodies, showed conclusively that the induced 569 and 5/B penicillinases were immunologically distinct.

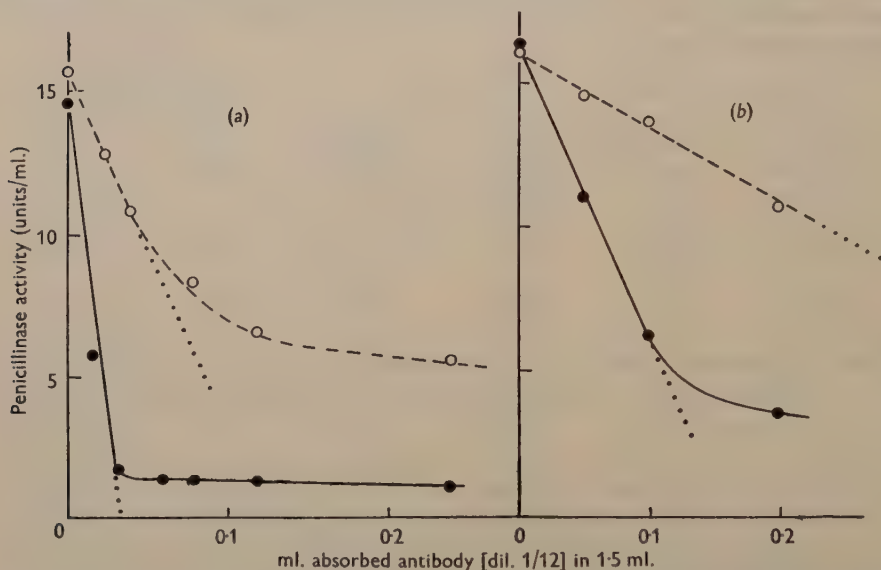


Fig. 3. Neutralization of penicillinase activity by anti-569 penicillinase antibody (362) solution partially absorbed by mixing 0.25 ml. with (a) 5/B penicillinase (4300 units) and (b) induced 569 penicillinase (8500 units). After removal of the precipitate, the supernatant fluid was titrated for neutralizing activity against both induced 569 (—●—●—) and 5/B (- - -○- - -) penicillinases. See text.

Partial absorption of antibodies. When enough enzyme (either induced 569 or 5/B) was added to the anti-569 antibody preparation to decrease its neutralizing titre by about 70 %, it was found that relative neutralization titres against induced 569 and 5/B—expressed as a simple ratio—rose from 1.4 to 2.6, and finally (with 90 % absorption) to 4.0. This finding is illustrated in Fig. 3, where the neutralization curves of anti-569 (362) antibody against induced 569 and 5/B are compared after partial absorption of antibodies (a) with 5/B, (b) with induced 569 penicillinase. These curves should be compared with those shown by the same antibody preparation before absorption (Fig. 2a (i)). As well as a three-fold increase in the anti-induced 569/anti-5/B neutralizing ratio, there is, as seen in the case of the 5/B-absorbed antibody preparation, a marked qualitative change in the anti-5/B neutralization curve which shows higher residual activity in excess antibody and a departure from

linearity after only 25 % of the enzyme had been neutralized (compared with about 80 % with unabsorbed serum). The enzyme neutralization titres of these absorbed antibody preparations have been calculated as maximum titres—i.e. in the presence of sufficient excess enzyme for there to be direct proportionality between enzyme neutralized and antibody added (linear portion of the neutralization curve).

It was also found that the antibody remaining after 90 % reduction in neutralizing titre by absorption with 5/B, failed to give a precipitate with 5/B enzyme. However, the unabsorbed antibody solution, diluted to the same extent (as judged by neutralization titre) in normal rabbit γ -globulin to give the same total γ -globulin concentration as in the partially absorbed antibody solution, gave an obvious precipitate when mixed with the same quantity of 5/B enzyme.

It thus appears that partially absorbed antibody differed qualitatively as well as quantitatively from unabsorbed antibody in that (a) the shape of the neutralization curve with the heterologous enzyme was quite different, (b) it was relatively much less efficient in neutralizing the heterologous enzyme as compared with the homologous enzyme and (c) although able to neutralize, and therefore to combine with, the heterologous enzyme, it failed to form a visible precipitate.

These results show that the induced 569 penicillinase differed significantly from the 5/B penicillinase. It might, however, still be argued that if the two enzymes were immunologically heterogeneous, consisting of two or more types of molecule, they might differ only in the proportions of different types present, and not in the types themselves; and it could be maintained that this is not a true immunological distinction. Even if this were admitted it would still be a rigidly consistent difference, because preparations of induced 569 penicillinase of varying degrees of purification and from cultures grown in different media and on different occasions have always given similar neutralization curves with partially absorbed antisera; and the same is true for preparations of 5/B penicillinase. But it must be conceded that if the antibodies evoked by a heterogeneous enzyme preparation were heterogeneous in a complementary fashion, the same sort of results as those described above with partially absorbed antibody might be expected. It might indeed be difficult formally to disprove this hypothesis, but it can be shown to be very unlikely by the experiments illustrated in Fig. 4. If, in fact, the enzymes really were immunologically heterogeneous it should be possible to absorb a proportion of enzyme molecules by precipitation with a limiting quantity of antibody (in a manner, strictly analogous to partial absorption of antibody with enzyme) and to obtain a selected sample of those enzyme molecules which were least 'efficient' in combining with antibody. It would then be surprising if such a sample of 'residual enzyme' did not react differently from the untreated enzyme in the neutralization reaction with antibody. However, Fig. 4 shows that a sample of crystalline 5/B penicillinase, after removal of 80 % of activity by precipitation with anti-569 antibody, did not differ significantly from untreated 5/B enzyme in its neutralization curve with anti-569 antibody (partially

absorbed with 5/B penicillinase in order to provide optimal conditions for disclosing a difference). It seems therefore reasonable to conclude that there is a true qualitative immunological difference between the induced 569 and the 5/B penicillinases.

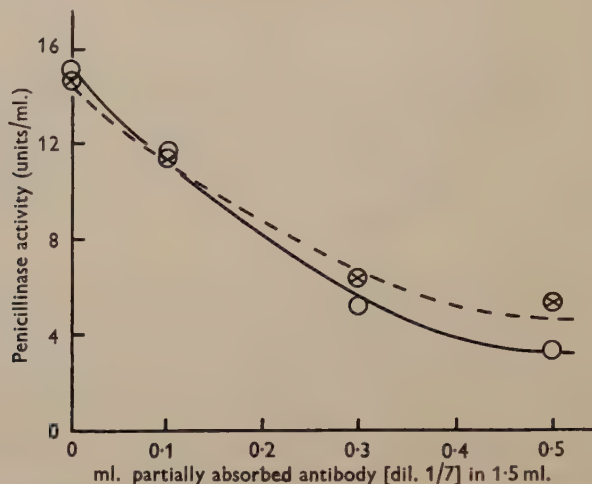


Fig. 4. Neutralization of penicillinase activity of (a) untreated 5/B enzyme (—○—○—) and (b) 5/B enzyme, reduced in activity by 80 % after partial precipitation with anti-569 (362) antibody (---⊗---⊗---), with anti-569 (362) antibody previously partially (75 %) absorbed with 5/B penicillinase. The '80 % precipitated' enzyme was prepared by mixing 2000 units of crystalline 5/B penicillinase with 0.06 ml. anti-569 (362) antibody γ -globulin solution in a final volume of 3.0 ml. physiological saline containing 0.01 M-phosphate (pH 7.0) incubating the mixture for 16 hr. at 35°, and centrifuging off the precipitate. 20 % of the original enzyme activity was found left in the supernatant fluid and this was compared with untreated 5/B penicillinase in the neutralization reaction with partially absorbed antibody.

Effect of antiserum on penicillin sensitivity

In spite of the fact that the penicillin resistance of both strain 5/B and strain 569 of *Bacillus cereus* is due in large measure to their ability to form penicillinase (see Sneath, 1955), Housewright & Henry (1947) found that addition of their anti-penicillinase serum to cultures of *B. cereus* strain 569 caused only a slight increase (from 100 to 50 units penicillin/ml. as minimum inhibitory concentration) in penicillin sensitivity. In this work, the addition of 5000 N.U. of anti-569 γ -globulin/ml. to broth inoculated with 10^8 spores of strain 569 was not found to lower significantly the minimum penicillin concentration needed to prevent growth. Similar negative results were obtained with spores of strain 5/B inoculated into penicillin-nutrient agar with and without the addition of 500 N.U. anti-569 γ -globulin/ml.

Applications

Immunological comparison of induced and constitutive penicillinases. The clear differentiation between the closely related 5/B and induced 569 penicillinases, provided by neutralization tests with partially absorbed antibody

preparations, suggested that this technique might be used to look for possible differences between the 569 and 569/H penicillinases described above. In work reported elsewhere (Pollock *et al.* 1956; Kogut *et al.* 1956) the interest of comparing the properties of penicillinases produced before and after induction by penicillin in the same strain, and without induction in a constitutive strain, has been fully discussed in relation to the mechanism of enzyme induction. Previous work (Manson, Pollock & Tridgell, 1954) had already failed to reveal any differences between basal and induced 569 penicillinase in neutralization tests with unabsorbed anti-569 serum. Although basal

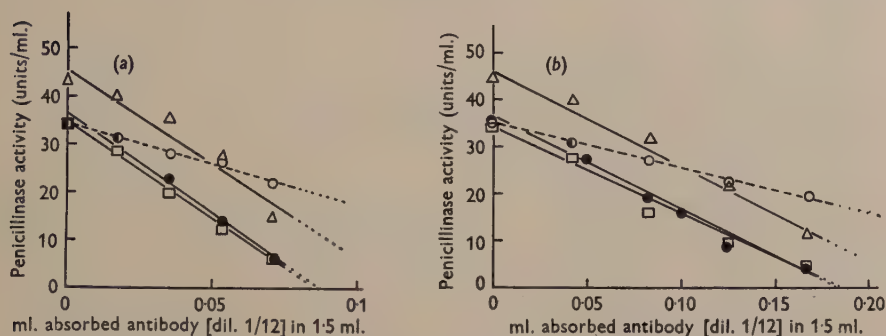


Fig. 5. Neutralization of penicillinase activity by anti-569 penicillinase antibody (362) solution partially absorbed by mixing 0.25 ml. with (a) 5/B penicillinase (3200 units) and (b) basal 569 penicillinase (5000 units). After removal of the precipitate, the supernatant fluid was titrated for neutralizing activity against basal 569 (\triangle — \triangle —), induced 569 (\bullet — \bullet —), 569/H (\square — \square —) and 5/B (\circ — \circ —) penicillinases. See text and Table 5.

enzyme production is so low that a purified preparation was not available, there was, nevertheless, sufficient of an impure preparation to permit neutralization and absorption tests. Accordingly, neutralization curves of all these 569 enzymes were obtained before and after partial absorption of antibody with each enzyme. The results are summarized in Table 5 and two examples of the families of neutralization curves so obtained are illustrated in Fig. 5 *a, b*. It can be seen that: (1) antibody, partially absorbed with any one of the four enzyme preparations, had the same neutralization titre when tested against the two 569 and the 569/H enzymes (though a different titre against 5/B); (2) although in Expt. I there were some differences in the residual antibody neutralization titres after partial absorption with an enzymically equivalent quantity of the 569 and 569/H penicillinase preparations, they corresponded to an apparent variation of only -11 to $+15\%$ about the mean in the amount of anti-569 antibodies removed, while in the second experiment the residual titres were almost identical; (3) the ratio of neutralization titres: anti-569/anti-5/B, increased from about 1.4 (for unabsorbed antibody) to 2.6 with 5/B-absorbed antibody. The significant point is not so much the total amount of antibody absorbed by the three different enzyme preparations (small variations being accounted for by relatively slight inaccuracy in estimation of the

Table 5. *Partial absorption of anti-569 penicillinase immune γ -globulin with different penicillinase preparations; and the increased resolving power shown by residual antibody in penicillinase-neutralization tests*

The enzyme preparation and antibody solution were mixed in a final volume of 3.0 ml. physiological saline containing 0.01 M-phosphate (pH 7.0) incubated at 35° for 16 hr., the precipitate centrifuged down and the supernatant fluid titrated for neutralizing ability against the different penicillinases.

Amount (ml.) of anti-569 (362) antibody solution used	Absorbing penicillinase		Residual neutralization titre (total neutralization units) of antibody against penicillinase				Ratio anti-induced 569: anti-5/B neutralization titres	
	Type	Amount units	Wt. (μ g.)	Neutralization units				5/B
				Basal 569	Induced 569	569/H		
0.25		0	Experiment 1					
		0	5430	6250	6910	4460	1.40	
0.25	Basal 569	(unabsorbed antibody)						
0.25	Induced 569	5000	1040	1040	1002	442	2.37	
0.25	Induced 569	5000	1865	1700	2004	1240	1.37	
0.25	Induced 569	5700	—	1420	1420	598	2.37	
0.25	Induced 569	6200	—	1018	—	450	2.27	
0.25	Induced 569	8500	—	152	—	40	3.80	
0.25	569/H	5000	2770	2810	2920	1750	1.60	
0.25	5/B	3200	2120	2140	2160	818	2.62	
0.3		0	Experiment 2					
		0		7500				
0.3	Basal 569	(unabsorbed antibody)						
0.3	Induced 569	5100	.	2470	.	.	.	
0.3	569/H	5100	.	2740	.	.	.	
0.3	5/B	3500	.	2590	.	.	.	
		15.7	.	4240	.	.	.	

quantity of enzyme added), but the fact that the residual antibody—which might be expected, from its relative neutralizing activity on 5/B and induced 569 penicillinase, to be capable of differentiating between two similar but distinct enzymes—showed in all cases the same neutralizing action against the 569 and 569/H penicillinases. Thus, by the immunological techniques employed, these three enzymes must be considered indistinguishable from each other.

Search for other material, in Bacillus cereus strain 569, immunologically related to penicillinase. It can be firmly concluded from the above results that there was no substance present as impurity in the crude basal 569 penicillinase preparation capable of combining with anti-569 neutralizing antibody apart from the penicillinase itself. Such a substance might, however, have been specifically excluded by the isolation procedure employed and its presence either within the cells or in the surrounding media had to be seriously considered in view of the discovery, by Cohn & Torriani (1953) in *Escherichia coli* of the protein Pz, immunologically and metabolically related to β -galactosidase. In order, therefore, to discover whether or not uninduced *Bacillus cereus*, strain 569, produced any enzymically inactive protein, immunologically related to penicillinase, analogous to the Pz protein of *E. coli*, a cell-disintegrate and an exocellular protein concentrate were prepared as follows. A 500 ml. culture of strain 569 in casein hydrolysate + citrate medium (see Kogut *et al.* 1956) was incubated without addition of penicillin at 35° on a shaker until a concentration of organisms equivalent to 0.75 mg. dry wt. of bacteria/ml. was reached. Oxine (8-hydroxyquinoline) at a final concentration of 8×10^{-4} M was added to prevent further enzyme production, and the cells separated by centrifugation, washed once in 8×10^{-4} M-oxine containing 10^{-2} M-phosphate buffer (pH 7.0) and resuspended in 3.0 ml. of the same oxine + buffer solution. The organisms were then disintegrated by crushing at -30° in a Hughes press. A 250 ml. sample of the culture supernatant fluid was concentrated by evaporation at 37° under reduced pressure to about 50 ml. in the apparatus of Craig (1950) and then further concentrated to 2.0 ml. by dialysis, under pressure, against 10^{-3} M-phosphate (pH 7.0). The sac was then tied off and the contents further dialysed against three changes of 500 ml. of the 10^{-3} M-phosphate buffer. The solution in the sac, which contained a small amount of insoluble matter, was made up to 7.5 ml. with the phosphate buffer and the 'protein' (i.e. non-dialysable N) content estimated by the Kjeldahl method (Ma & Zuazaga, 1942). Samples of the disintegrated cell preparation and of the exocellular concentrate of non-dialysable material and of purified induced 569 exopenicillinase were then each mixed with 0.25 ml. anti-569 (362) antibody solution. After overnight incubation at 35°, insoluble material was centrifuged off and the supernatant fluids tested for neutralizing activity against induced 569 penicillinase (see Table 6). The amounts of cell-disintegrate and exocellular concentrate used were adjusted to correspond to the product of exactly ten times the quantity of cells which formed the amount of pure induced 569 penicillinase added (itself adjusted so as to absorb nearly all the anti-penicillinase antibodies present). This was done simply in order to increase the significance of a negative result.

It can be seen from Table 6 that there was only a very slight diminution in antibody titre after treatment with the two preparations from uninduced cells, and it can be concluded with reasonable certainty that there is no appreciable amount of any substance produced by uninduced *Bacillus cereus*, strain 569 capable of absorbing (and therefore probably combining with) penicillinase-neutralizing antibodies, apart from penicillinase itself.

Table 6. *Absorption of anti-penicillinase antibodies by different fractions from Bacillus cereus strain 569*

0.25 ml. lots of anti-569 (362) antibody solution were mixed with the 'absorbing material' in a final volume of 3.0 ml. of saline containing 0.01 M-phosphate (pH 7.0). After incubation at 35° for 16 hr. the insoluble material was centrifuged off and the supernatant fluid titrated for neutralizing ability against induced 569 penicillinase.

'Absorbing' material	Quantity added (mg.)	Dry wt. of bacteria by which material was formed (mg.)	Total penicillinase activity (units)	Total neutralization titre of antibody		Neutralizing antibody removed (%)
				Before absorption	After absorption	
				Neutralization	units	
Purified exopenicillinase (from induced cells)	0.023	4.5	8000	7500	455	94
Disintegrated uninduced cells	45	45	2	7500	7050	6
Exocellular concentrate from uninduced cells	1.07	45	76	7500	6700	10.6

DISCUSSION

Absorption of anti-569 (362) antibody preparation with either 569 or 5/B penicillinase removes all, or very nearly all, neutralizing antibodies to both 5/B and 569 enzymes. All the antibody molecules must therefore be capable of combining with both enzymes and it seems to be out of the question that they consist of mixtures of two kinds of antibodies each exclusively directed towards one or other type of penicillinase. Neutralization titres of both anti-569 and anti-5/B unabsorbed sera against 5/B and 569 enzymes differ, at the most, by only 40 %. Indeed, since the specific activity of induced 569 penicillinase is, on a weight basis, 37 % higher, or on a molecular basis, 20 % higher (Kogut *et al.* 1956), than that of 5/B, it almost appears as if a given amount of antibody combined with the same number of antigen molecules—or at least with the same weight of antigen—in both cases. Nevertheless, partial absorption of antibody with either 5/B or 569 penicillinase leaves a residual antibody fraction able to neutralize four times as much 569 penicillinase activity as 5/B. Although at first sight this may appear surprising it can be understood on the hypothesis that a proportion of antibody evoked by 569 penicillinase is non-specifically 'inefficient' in the sense that the molecules react more slowly, and with less neutralizing power than the average, with both types of penicillinase. These 'inefficient' antibodies would be selectively left behind on partial absorption with either 569 or 5/B enzyme, but might nevertheless be expected to be relatively more efficient against the homologous antigen. The picture

would fit well the concept of an 'antibody array' described by Fulton (1953), which visualizes a heterogeneous population of antibody molecules showing a continuous spectrum of antigen-combining properties (e.g. closeness of fit, speed of reaction, etc.). Thus, on the assumption that the antigen-antibody reaction is irreversible, the antibody molecules remaining after nearly complete absorption with antigen might be considered as the most slowly reacting of all amongst a heterogeneous population with widely differing speeds of combination.

In contrast to this non-uniformity of the antibody population, no evidence was found to support the possibility of immunological heterogeneity amongst the enzyme molecules. The residual enzyme activity after precipitation of 80 % of a sample of crystalline 5/B penicillinase with antibody, was indistinguishable from unabsorbed 5/B penicillinase in the neutralization reaction with a partially absorbed anti-569 antibody preparation.

In general, antibodies which neutralize the activity of the enzyme used as immunizing antigen are quite inactive against the same type of enzyme obtained from a different source. But cross-neutralization reactions can occur with very closely related enzymes (Miles & Miles, 1950: *Clostridium lecithinases*; Kirk & Sumner, 1932: Jack and Soy bean ureases) and have been studied in some detail by MacFarlane (1950) who found that *Clostridium haemolyticum* and *C. oedematiens* lecithinases were both inhibited by antiserum produced in rabbits immunized with either enzyme, but their neutralization slopes (though indistinguishable with anti-oedematiens lecithinase serum) differed by about 100 % with anti-haemolyticum lecithinase serum. Cross-precipitation reactions between enzymes from different sources have been more frequently reported. One of the most accurately studied and interesting is that between horse and beef catalase reported by Campbell & Fourn (1939) who found that twice as much anti-beef catalase serum was needed to precipitate a given quantity of horse catalase as was needed for precipitation of the same amount of homologous catalase. Thus, although in the case of penicillinase an immunological difference between the induced 569 and the 5/B enzymes was unmistakably disclosed by partial antibody absorption tests, the results of direct neutralization tests indicate a closer relationship than that shown by the ureases, lecithinases and catalases from different species similarly investigated.

By the same argument, the failure to detect any differences between the 569 and 569/H penicillinases with preparations of an antiserum partially absorbed with each type of enzyme must be considered as good evidence in support of their identity. If, at least, there are any differences between basal and induced 569 and 569/H penicillinases they must be restricted to parts of the molecule not involved in the combination with, or neutralization by, antibody. This result is consistent with an independent physico-chemical comparison between purified 569 induced and 569/H penicillinases (Kogut *et al.* 1956) which revealed no significant differences in electrophoretic mobility, specific activity, sedimentation constant, diffusion coefficient or solubility in ammonium sulphate, while the 5/B enzyme could be shown to be quite distinct

from the other penicillinases by these criteria. The fact that the basal penicillinase formed by *Bacillus cereus*, strain 569 without penicillin is immunologically indistinguishable from the induced penicillinase produced by the same strain after treatment with penicillin gives further support to the hypothesis (previously based largely on kinetic and physico-chemical data) that the mechanisms of their formation, in the absence and presence of inducer respectively, are fundamentally similar.

Fig. 2 shows that there is a variation from 30 to 97 % in the extent to which a penicillinase may be neutralized by excess antiserum. The relative importance of the source of enzyme, method of immunization and individual variation of animals in determining the maximum extent of neutralization has not been studied in detail. However, the difference between antisera from rabbits 1 and 2 (immunized with the same enzyme preparation and by exactly the same technique)—Fig. 2*b* (i) and (ii)—shows that individual variation in the immunized animal may be an important influence. It is clear that other factors, apart from properties associated with the type of enzyme concerned, may determine the degree of neutralization due to combination with antibody.

Attempts to reveal the presence of a substance formed by uninduced *Bacillus cereus*, strain 569, bearing the same immunological relationship to penicillinase as the 'Pz' protein does to induced β -galactosidase in *Escherichia coli* (Cohn & Torriani, 1952) were unsuccessful; but they do not of course exclude the possibility of there being other proteins related to penicillinase in a manner not demonstrable by the techniques used. The results summarized in Table 6 show conclusively that no substance was formed by strain 569 in significant quantities, capable of combining with penicillinase-neutralizing antibodies other than penicillinase itself; and demonstrate the high specificity of the reaction between enzyme and antibody.

The results reported here have been based almost exclusively on a study of the neutralization reaction between enzyme and antibody. The method is economical of both time and material and, at least in this instance, is unaffected by the presence of other substances (which may complicate the interpretation of the precipitation reaction). No doubt quantitative analyses of antigen-antibody precipitates, along classic immunological lines, would give further valuable information.

An interesting and somewhat similar immunological analysis has, indeed, been carried out by Markert & Owen (1954) and Owen & Markert (1955) working with tyrosinase in extracts of *Glomerella* sp. By quantitative precipitation tests (to determine 'equivalence points') they concluded that the tyrosinases from the wild type and a number of mutant strains producing the enzyme at widely differing rates, were immunologically indistinguishable; and that there were no substances present in the extracts capable of combining with anti-tyrosinase antibodies other than tyrosinase itself. It is, perhaps, doubtful whether their technique would have allowed them to recognize a difference as fine as that between the 569 and 5/B penicillinases. But their findings are in accordance with those reported here and elsewhere (Kogut *et al.* 1956) on penicillin-induced and spontaneous mutational changes in rates of

penicillinase production by *Bacillus cereus*, strain 569, which also indicate that the changes observed are purely quantitative (at a molecular level), the types of enzyme proteins formed in all cases being apparently identical.

I am deeply indebted to Dr Melvin Cohn for much stimulating and helpful advice at the beginning of this work. I am grateful to Dr J. H. Humphrey and Dr Forrest Fulton for discussing the work with me in detail, to Dr P. H. A. Sneath for preparing the anti-5/B penicillinase sera and to Miss Joan Fleming for technical assistance.

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Invertase Formation in *Saccharomyces fragilis*

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SUMMARY: The formation of invertase in *Saccharomyces fragilis* was studied by the continuous culture technique. Invertase formation was markedly inhibited by glucose in the culture medium at concentrations greater than 0.001 % (w/v), by galactose and lactose at all concentrations tested, and by several other carbon sources. The mean generation time of the organisms and the concentration of ammonia and growth factors had no significant effect on invertase formation. The pH value of the growth medium modified the amount of enzyme formed. The invertase activity, together with non-enzymic hydrolysis of sucrose by the acidic culture medium, was sufficient to account for the disappearance of the observed amount of sucrose from media containing sucrose.

During the growth of a micro-organism under ordinary culture conditions in a limited volume of medium the growth rate continually changes and the chemical composition of the medium varies as a result of the metabolism of the organism. In such circumstances it is difficult, if not impossible, to assess all the factors which control the enzymic activities of the cells. However, if a steady state could be achieved so that known environmental factors could be kept constant, it would be considerably easier to investigate the effect of each such factor. One way of achieving a culture in a steady state is by use of the continuous culture technique described by Monod (1950) and by Novick & Szilard (1950 *a, b*), in which the growth rate is determined by the concentration of one particular constituent of the growth medium. Under such conditions the effects of other constituents on enzymic activity can be studied, uncomplicated by changes in growth rate. Also, by altering the rate of addition of the medium, and hence of the limiting factor, the growth rate can be varied without appreciably affecting the steady state concentration of other substances in the growth medium. Therefore, it should be possible to determine whether growth rate *per se* has any effect on enzyme formation.

There have been many reports of variations of the enzymic activities of bacterial and yeast cells with growth time. Wooldridge, Knox & Glass (1936) found that the dehydrogenase activities of several bacterial species were low in 'young' cultures, increased with continued growth and then decreased again. Gale & Stephenson (1938) reported a similar effect with serine deaminase in *Escherichia coli*. Gale (1940) found that the activities of the bacterial amino-acid decarboxylases were highest when the culture density was maximal. Similar results were obtained with aspartase in *E. coli* (Gale, 1938) and were shown to be due to a change in the chemical composition of the medium, for when the medium in which a culture had grown was sterilized and re-inoculated, the aspartase was high at all stages of growth. Davies (1953) found that

when *Saccharomyces fragilis* was grown in a medium containing 2% (w/v) glucose, the invertase activity was low during the early phases of growth but later increased rapidly. When the glucose concentration was increased to 10% (w/v), invertase activity was low at all stages of growth. The author concluded from these and other experiments that the presence of glucose in the medium inhibited the formation of invertase. Invertase synthesis was demonstrated in suspensions of washed organisms and was inhibited by glucose and fructose.

The present work describes the effect on invertase formation in *Saccharomyces fragilis* of certain sugars and other carbon sources. The effects of the mean generation time of the organism, concentrations of ammonium and hydrogen ions and of growth factors have also been studied. Most of this work was carried out by means of the continuous culture technique.

METHODS

The chemostat. Fig. 1 shows the most satisfactory type of chemostat devised for this work. It resembles the chemostat of Novick & Szilard (1950*a*), the main difference being in the aerator and the medium feed. The aerator has the dual functions of aerating and stirring the medium. The flow of air through the aerator was sufficiently great to set up an atomizing action, sucking culture fluid in through the side opening and expelling it together with fresh medium at the base. Standard taper (B 24) ground glass joints at 'A' and 'B' enabled the cap and the aerator to be removed to facilitate cleaning; 'B' also served as the inoculation port. The medium was supplied by a Bayliss and Muller type peristaltic pump (Booth & Green, 1938) driven from a 0.125 h.p. constant speed induction motor through a train of gears. Alterations in the pumping rate are achieved by changing the gear ratio. The pump rollers and rubber tubing were lubricated with silicone grease. The variation in flow rate with a fixed gear ratio was $\pm 0.5\%$.

Organisms. *Saccharomyces fragilis* Jørgensen was obtained from the Centraalbureau voor Schimmelcultures, Delft, and *Torulopsis pulcherrima* from the National Collection of Type Cultures, Elstree, in 1946.

Media. The basal medium was that of Davies, Falkiner, Wilkinson & Peel (1951) with Difco yeast extract replaced by the following amounts of growth factors ($\mu\text{g./ml.}$): biotin, 0.01; pantothenic acid, nicotinic acid, pyridoxine, thiamine, each 1.0; inositol, 5.0. The basal medium (less thiamine) was

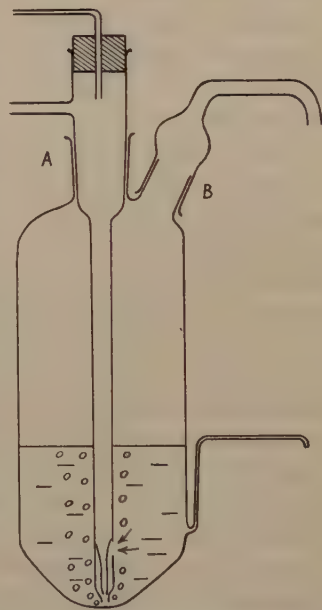


Fig. 1. Diagram of chemostat.

sterilized by autoclaving. The various carbon sources, glucose, galactose, glycerol, sodium acetate, sodium citrate, sodium lactate, and sodium succinate were autoclaved separately; sucrose, lactose and thiamine were sterilized by filtration; ethanol was added as absolute ethanol. The carbon source and thiamine were added aseptically to the basal medium and sterile water added to replace that lost by evaporation.

For chemostat experiments the basal medium was made up in graduated aspirator bottles. For static cultures the medium was dispensed into Roux bottles (150 ml./bottle) and for shaken cultures into 500 ml. Erlenmeyer flasks (150 ml./flask). The technique for studying variations in enzymic activity with time of growth was that described by Davies (1953). The inoculum was grown for 24 hr. in boiling tubes containing the basal medium + 2% (w/v) glucose, except for the experiments where growth took place on ethanol, glycerol, sodium acetate, sodium citrate, sodium lactate or sodium succinate, in which cases the glucose was replaced by the respective carbon source. The growth temperature was 30°.

Chemostat procedure. Monod (1950) showed that under steady state conditions of growth in a chemostat, the rate of addition of medium (or washing-out rate) determines the mean generation time of the organisms. The relationship can be expressed as follows: Mean generation time = $(\log_e 2)/\text{washing-out rate}$. The minimum value for the mean generation time is determined by the composition of the medium and the gas phase and increases in the washing-out rate beyond this point result in dilution out of the culture to vanishing point.

In practice the chemostat was connected aseptically to the medium reservoir, the air supply turned on, the chemostat filled with medium and inoculated. After 15–20 hr. the addition of medium was begun at the lowest practicable rate (10 ml./hr.). It was determined that after a further 24 hr., steady state conditions prevailed and the first sample was collected in a receiver cooled in ice. The flow rate was then increased to the value chosen for the next sample which was collected on the following day. This procedure was continued until the lowest practicable mean generation time of 1.5 hr. was attained.

Organism suspensions. The yeast was centrifuged from the growth medium, washed twice with distilled water and re-suspended in distilled water. Suspension density was determined by drying a sample at 105° to constant weight. This procedure was adopted because it was found that the ratio of turbidity to dry weight could vary over a twofold range depending on how the yeast was grown.

Invertase activity was estimated by the method of Davies (1953), which uses cetyltrimethylammonium bromide. The unit of activity has been defined as the amount of enzyme which hydrolyses 1 μ mole sucrose in 1 hr. at 25°. From six replicate samples collected from a chemostat running at a fixed flow rate it was found that the coefficient of variation was less than 5% when the invertase activity was greater than 100 units/mg. When the activity was low (< 2 units/mg.) the absolute variation between replicate samples was less than 0.5 unit/mg.

Analysis of growth media. Ammonia was determined by steam distillation in a micro-distillation apparatus (Markham, 1942) followed by nesslerization (Folin & Wu, 1919). Sugars were determined on 0.04–1.8 ml. samples by fermentation in Warburg manometers according to the method of Reithel (1951) modified by increasing the concentration of sodium azide to 1.3×10^{-3} M. Yeast was grown for 24 hr. in Roux bottles containing 150 ml. of the basal medium of Davies *et al.* (1951) and an appropriate sugar at a concentration of 2% (w/v). The yeast was harvested as described above and suspended in 0.17 M-potassium phosphate buffer (pH 6.0) to give a cell density of c. 100 mg./ml. Sodium azide was added to a concentration of 4×10^{-3} M and the preparation was shaken for 2 hr. at 25° to eliminate endogenous fermentation. Each manometer cup contained 1 ml. of this preparation in a final fluid volume of 3 ml. Carbon dioxide evolution at pH 6.0 was 95–98% of the theoretical 2 mole CO₂/mole hexose for glucose, sucrose and lactose, and 92–95% for galactose.

For determination of glucose and sucrose, *Saccharomyces fragilis* was grown on a medium containing glucose. For determination of galactose and lactose *S. fragilis* was grown on a medium containing galactose. For determination of invert sugar in cultures containing sucrose *Torulopsis pulcherrima* was grown on a medium containing glucose.

Chemicals. Kerfoot's Biochemical Reagent Sugars were used throughout; other chemicals were of analytical quality.

RESULTS

Invertase formation during growth in glucose media in shaken flasks

The change in invertase content of the cells with time of growth under various conditions is shown in Fig. 2*a, b*. Curves marked *A* show invertase formation and growth in a medium containing an initial concentration of 1.5% (w/v) glucose, 352 mg. ammonia-N/l. and 20% of the standard amount of growth factors. The invertase content of the cells remained low during the greater part of growth and then rose rapidly to high values. This rise in activity was concurrent with the disappearance of glucose from the medium. For curves marked *B* the medium initially contained 0.1% (w/v) glucose. Growth ceased earlier and at a lower cell density than in the previous case. The invertase activity was again low in the early phases of growth. Curves *C* and *D* (Fig. 2*b*) show the effect of decreasing the amount of nitrogen in the medium; in both cases growth ceased before glucose was fully utilized and the invertase activity was low throughout the experiment. Growth started at approximately the same rate in both cases, but the final yield of organisms was lower in the medium containing the smaller amount of nitrogen. Curves *D* and *E* show the effect of alterations in the amount of growth factors, while glucose and nitrogen are kept constant. The growth curves were almost identical, but whereas in *D* the invertase content remained low, with the smaller concentration of growth factors (curve *E*) glucose utilization continued after growth ceased and invertase activity increased concomitantly. The reason for this is not understood.

Invertase formation during growth in the chemostat

Effect of glucose concentration and mean generation time. When the yeast was grown in a chemostat supplied with medium initially containing 9.5% (w/v) glucose, 105 μ g. ammonia-N/ml. and standard concentrations of growth factors, the glucose concentration in the culture became steady at approximately 8% (w/v), the nitrogen concentration at approximately 20 μ g./ml. and

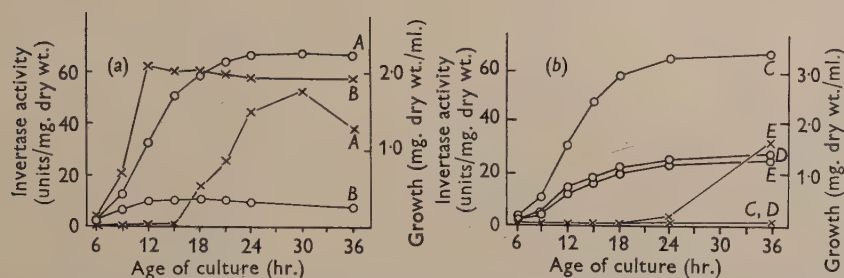


Fig. 2. The effect on invertase content of growth for different periods of time in media containing various amounts of glucose, nitrogen and growth factors. $\times-\times$, invertase activity; $\circ-\circ$, culture density. The glucose concentrations in the cultures at the different times are shown in the table. Curve A: 1.5% (w/v) glucose, 352 mg. ammonia-N/l., 20% of standard growth factors. Curve B: 0.1% (w/v) glucose, otherwise as curve A. Curve C: 2.0% (w/v) glucose, 352 mg. ammonia-N/l., standard amounts of growth factors. Curve D: 105 mg. ammonia-N/l., otherwise as curve C. Curve E: 20% of standard growth factors, otherwise as curve D.

Medium	Residual glucose (% w/v) at various times (hr.)						
	6	9	12	15	18	24	36
A	1.2	1.0	0.64	0.24	0.03	Nil	Nil
B	0.05	0.01	Nil	Nil	Nil	Nil	Nil
C	1.8	1.6	1.3	0.55	0.26	0.15	0.14
D	1.8	1.7	1.5	1.4	1.3	1.2	1.2
E	1.8	1.7	1.6	1.4	1.2	0.57	Nil

the invertase activity of the resultant organisms was less than 0.6 unit/mg. at all values of the mean generation time. Low degrees of activity were also observed when the supplied medium contained 2% (w/v) glucose; in this case the glucose concentration in the culture was always greater than 0.5% (w/v); the nitrogen concentration was less than 3 μ g./ml. for all flow rates except that corresponding to a mean generation time of 1.6 hr. when it rose to 7 μ g./ml.

Fig. 3 shows the invertase activities and culture glucose concentrations for growth in media initially containing 1.0, 0.5 and 0.25% (w/v) glucose. In these cases the activity varied with the mean generation time, but could also be correlated with the glucose concentrations in the cultures. In Fig. 4, which is compiled from the results of a large number of experiments, the invertase activity is plotted against the logarithm of the glucose concentration in the culture for mean generation times of 3 and 10 hr. The two curves are substantially the same, showing that the rate of cell division has little effect on

the biosynthesis of invertase under these conditions. It would appear that invertase formation only occurs to a significant extent in media containing less than 0.01 % (w/v) glucose.

Effect of growth factors and nitrogen concentration. Biotin, nicotinic acid and pantothenic acid are essential for growth of this strain of *Saccharomyces fragilis*; inositol, pyridoxine and thiamin are stimulatory (Bigger, 1952). It is possible to devise conditions in which nitrogen and glucose are in excess; this

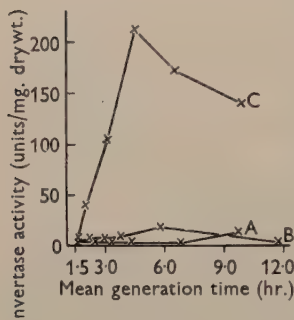


Fig. 3

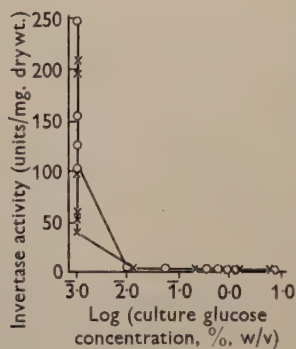


Fig. 4

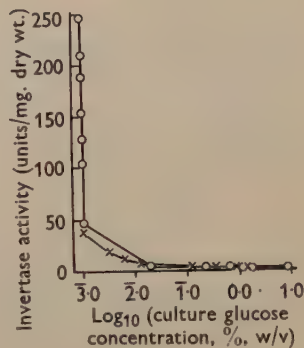


Fig. 5

Fig. 3. Variation of invertase activity with mean generation time. Initial glucose concentrations (% w/v) in media: A, 1.0; B, 0.5; C, 0.25.

Fig. 4. Effect of culture glucose concentration and mean generation time on invertase activity. x—x, cells grown with a mean generation time of 3 hr.; o—o, cells grown with a mean generation time of 10 hr. The limit of sensitivity of the sugar analysis procedure is 0.001 % glucose.

Fig. 5. Effect of culture glucose concentration and nitrogen concentration on invertase activity. x—x, culture containing < 2 µg. ammonia-N/ml.; o—o, culture containing > 10 µg. ammonia-N/ml.

can be done by making one or other growth factor limiting. Under these conditions it was found that neither variation in nitrogen concentration nor in the nature of the growth factor which was limiting, had any appreciable effect on the invertase activity. The overriding influence of the steady state glucose concentration in the culture is again shown by Fig. 5 in which the logarithm of the culture glucose concentration is plotted against the invertase activity for the two cases (a) where the nitrogen concentration is less than 2 µg./ml.; (b) where it is greater than 10 µg./ml. The two curves are almost identical at glucose concentrations greater than 0.001 % (w/v), which is the limit of sensitivity of the sugar determination procedure. This demonstrates clearly the absence of any significant effect of nitrogen concentration, irrespective of the nature of the growth-limiting substance.

Effect of pH value of growth medium. The pH value of the culture in the chemostat varied between pH 2.6 and 3.4, depending on the concentration of sugar in the medium. By adding appropriate amounts of potassium hydroxide to the medium it was possible to grow the yeast over a wide range of pH values

in media of otherwise similar composition. When the basal medium contained 0.1% (w/v) glucose and the mean generation time was 4 hr., the potential invertase activity (i.e. activity measured at the optimal pH value for invertase action) varied with pH value as shown in Fig. 6, curve *A*. Between pH 3.5 and 7.7 there was no detectable glucose in the culture; between pH 7.7 and 8.3 the glucose increased to 0.014% (w/v). The effective activity (see Gale & Epps, 1942) is also plotted, and was found to decrease steadily as the pH value increased from 3.5 to 8.3 (curve *B*).

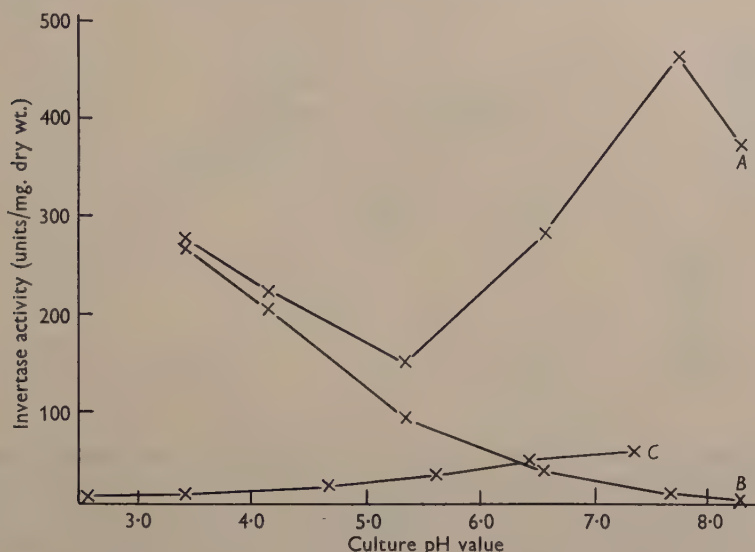


Fig. 6. Effect of culture pH value on invertase content. Curve *A*: potential activity of cells grown with a mean generation time of 4.0 hr. in a medium initially containing 0.1% glucose. Curve *B*: effective activity of the aforementioned cells. Curve *C*: potential activity $\times 10$ of cells grown with a mean generation time of 3.5 hr. in a medium initially containing 2.0% glucose. The potential activities were determined at the optimum pH value (3.7) and a correction made for acid hydrolysis in the test system.

When the yeast was grown in the basal medium plus 2% (w/v) glucose at a mean generation time of 3.5 hr., the potential activity increased from 0.6 unit/mg. at pH 2.6 to 6 units/mg. at pH 7.6 (Fig. 6, curve *C*), while the effective activity was approximately constant over the whole range.

Media containing sugars other than glucose

Sucrose. Fig. 7 shows that when growth occurred in a medium containing an excess of sucrose (1%, w/v, curve *A*) the invertase activity was low (0.5–2.0 units/mg.). Since the culture was found to contain both sucrose (0.3–0.6%, w/v) and invert sugar (0.02–0.05%, w/v), it was impossible to decide whether sucrose itself exerts any specific effect on the invertase activity. When the yeast was grown in a 0.2% (w/v) sucrose medium (curve *B*) the invertase activity was high. Although in this case the invertase activity

varied with mean generation time, it could again be correlated with changes in the sugar (mostly invert sugar) concentration in the culture (curve C). The growth obtained in both concentrations of sucrose was approximately the same as that obtained in corresponding media containing glucose.

When the yeast is grown in an excess of sucrose it is possible to compare the disappearance of sucrose from the medium with the maximum possible disappearance calculated on the basis of the observed invertase activity. The results are given in Table 1. It is evident that the sucrose disappearance can

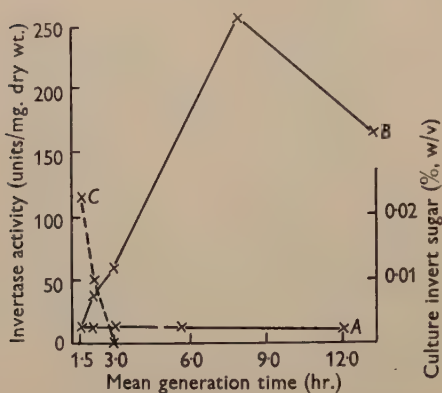


Fig. 7

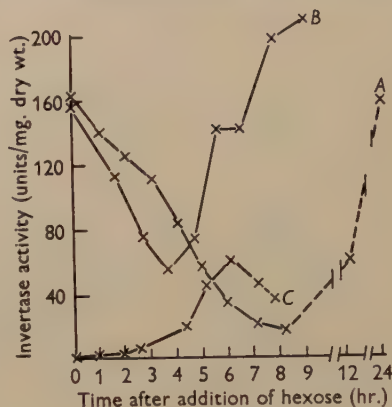


Fig. 8

Fig. 7. Effect of growth at different mean generation times in media containing sucrose. Curve A, invertase activities $\times 10$ during growth in a medium initially containing 1% (w/v) sucrose. Curve B, invertase activities during growth in a medium initially containing 0.2% sucrose. Curve C, culture invert sugar concentrations corresponding to curve B.

Fig. 8. Curves A and B: effect of adding galactose (1%, w/v, curve A; 0.1%, w/v, curve B) to cultures growing on 0.1% (w/v) glucose medium with a mean generation time of 3.5 hr. Curve C: effect of adding glucose (1.0%, w/v) to a culture growing on 0.2% (w/v) galactose medium with a mean generation time of 3.7 hr., the activities are shown times ten for clarity.

Table 1. Correlation between observed sucrose disappearance in media containing an excess of sucrose and that calculated from the invertase content

Enzymic hydrolysis measured at pH value and temperature of culture and at optimal sucrose concentration. Resulting values were then corrected to allow for the fact that the culture sucrose concentration was suboptimal, the correction factor being 0.9.

	Expt.		
	I	II	III
pH value of culture	3.15	3.19	3.12
Sucrose disappearance due to spontaneous hydrolysis at culture pH value (mg./ml.)	0.2	0.2	0.2
Sucrose disappearance due to enzymic hydrolysis (mg./ml.)	1.3	1.7	1.2
Observed sucrose disappearance (mg./ml.)	1.5	1.9	1.3

be accounted for by the invertase content of the yeast provided due allowance is made for non-enzymic hydrolysis of sucrose under the acidic conditions of the medium.

Lactose or galactose. In Table 2, the invertase activity of yeast grown in various concentrations of lactose or galactose is compared with that for glucose media. It will be seen that the response is different in that high degrees of activity were not obtained in low concentrations of lactose or galactose, in fact, on increasing the galactose concentration to 2% (w/v) the invertase activity rose fivefold to tenfold. Similar results were obtained for mean generation times between 1.6 and 10 hr.

Table 2. *Invertase activities of Saccharomyces fragilis grown in media containing various sugars*

Yeast grown in chemostat at a mean generation time of 3.3 hr. Invertase activities expressed as units/mg. dry weight.

Concentration of sugar in growth medium (% w/v)	Invertase activities for growth on		
	Glucose	Galactose	Lactose
2.0	0.2-0.6	4.0	—
1.0	0.2-0.6	0.6	1.4
0.2	200	0.2	0.7

Mixtures of glucose and galactose. The effect of adding galactose to an exponentially growing culture which had a high invertase activity was demonstrated by the following experiments. From a culture growing with a mean generation time of 3.5 hr. in the basal medium + 0.1% (w/v) glucose a 10 ml. sample was collected and then galactose was added directly to the culture to give a final concentration of 1.0% in the first experiment and 0.1% (w/v) in the second experiment. Further samples were collected at intervals and their invertase contents determined. There was an immediate and rapid decrease in the invertase activity followed by a rise (Fig. 8, curves *A* and *B*). The decrease was greater with 1% (w/v) galactose and the activity was low for a longer time, but the rate of decline in activity was greater with 0.1% galactose.

The effect of adding glucose (final concentration 1.0% (w/v)) to a culture growing exponentially with a mean generation time of 3.7 hr. on a 0.2% (w/v) galactose medium is also shown in Fig. 8, curve *C*. A rise in activity to 6 units/mg. was observed 6 hr. after addition of glucose. Table 3 shows the

Table 3. *Effect on invertase content of growth in media containing various amounts of glucose and galactose*

Saccharomyces fragilis grown in chemostat at a mean generation time of 3.3 hr. In all cases the culture hexose concentration was less than 0.001% (w/v).

Initial glucose concentration (% w/v)	0.20	0.18	0.15	0.11
Initial galactose concentration (% w/v)	Nil	0.02	0.05	0.09
Invertase activity (units/mg. dry wt.)	190	127	36	6.5

effect on invertase formation of growth in a medium containing various amounts of glucose and galactose and demonstrates clearly the inhibitory effect of galactose under these conditions.

Growth in other carbon sources. Under aerobic conditions *Saccharomyces fragilis* used arabinose, fructose, glucose, galactose, lactose, mannose, sucrose, raffinose, xylose, ethanol, glycerol, acetate, citrate, lactate or succinate for growth. Ribose, sorbose, dulcitol, mannitol, sorbitol and sodium potassium tartrate were not utilized. Except for the sugars, and for lactate for which the minimum mean generation time was 3.5 hr., the minimum mean generation

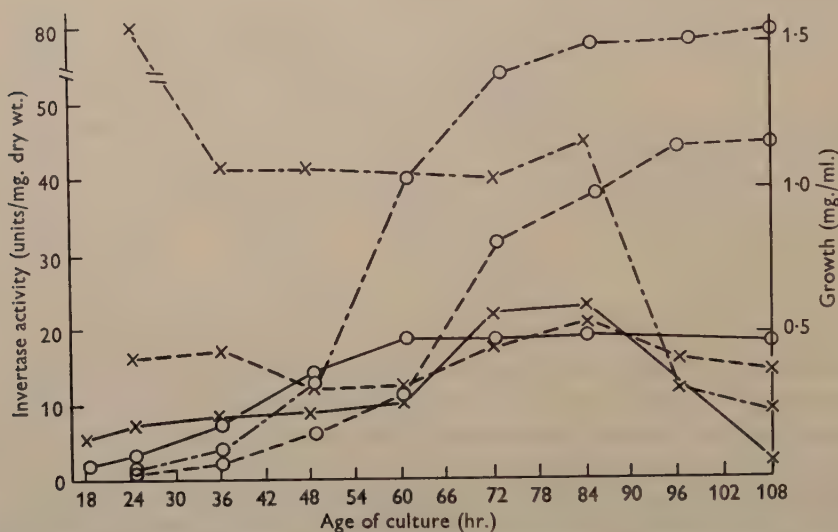


Fig. 9. Effect of 'age of culture' on the invertase content of *S. fragilis* grown on basal medium containing non-carbohydrate carbon sources. x, invertase activities; o, culture density; —, basal medium plus 1% (w/v) sodium succinate; - - -, basal medium plus 1.0% (w/v) ethanol; - · - ·, basal medium plus 1% (w/v) glycerol.

time exceeded 5 hr. and in some cases (acetate, citrate) was 8–12 hr. It was not practicable to use the chemostat for such low growth rates. Cultures were therefore grown in Roux bottles on basal medium containing 1% of various substances as carbon sources and the invertase activity determined after various periods of growth.

Acetate, citrate or succinate gave similar results up to the time when growth ceased. In the earlier stages of growth the invertase activity was about ten times that in a 1% (w/v) glucose medium, the activity then increased to a maximum towards the end of growth. With succinate, but not acetate or citrate, there was a fall in invertase activity shortly after growth had ceased (Fig. 9).

Ethanol at a concentration of 1% (v/v) gave an invertase activity of c. 15 units/mg. which showed little variation throughout growth.

With glycerol invertase activity was high (80 units/mg.) at the start of the exponential phase of growth but it rapidly fell to about 40 units/mg. at which

level it remained constant until after growth ceased when there was a further fall to 9 units/mg. (Fig. 9).

With lactate the invertase activity remained at a low level (0.1–1.0 unit/mg.) throughout the growth period.

DISCUSSION

The formation of many enzymes is suppressed by the presence of glucose in the growth medium (Gale, 1943). The effect on invertase formation, however, is wider than an inhibition by glucose, for if the invertase activity in the presence of low concentrations of glucose is taken as the uninhibited value, then all carbon sources tested are inhibitory, though with some, such as raffinose (Davies, 1953) and glycerol, the inhibition is small. The type of response shown by the yeast depends on both the nature and the concentration of the carbon source. For instance, the response to galactose differs from that to glucose; the highest activities (about 2% of that observed in a medium containing low concentrations of glucose) are found in media initially containing 2% (w/v) galactose, while lower invertase activities are observed when the galactose concentration is reduced. Yeast which has grown on a medium containing an excess of sucrose has a low invertase activity. At first sight this would seem to indicate that invertase formation is suppressed by sucrose. However, the low activity can be accounted for by the presence of low concentrations of invert sugar in the culture. Because of this, it is impossible under the conditions used to decide whether sucrose has any inhibitory or stimulatory action on invertase formation. The observed low concentrations of invert sugar in these cultures are in accordance with the hypothesis of Hestrin & Lindegren (1952), that biosynthesis of carbohydrases in yeast continues until the rate of production of hexose by hydrolysis of oligosaccharides slightly exceeds the rate of utilization of the hexose, the accumulating hexose then inhibiting further enzyme formation. The fact that, after allowance had been made for acid hydrolysis in the culture, sucrose disappearance in these cultures agreed closely with that calculated on the basis of the invertase activity of the organisms in washed suspension, would suggest that the sole pathway of sucrose metabolism is via hydrolysis to invert sugar.

Davies (1953) found that with sucrose as energy source synthesis of invertase by washed suspensions of *Saccharomyces fragilis* proceeded optimally between pH 4.0 and 5.0, the optimum pH value for enzyme action being approximately 4.0. However, the same relationship was observed when glucose was the energy source, in which case the energy supply is not dependent on the activity of the enzyme. In growing cultures the formation of invertase shows a different response to pH value, the potential activity of the yeast grown in a 0.1% (w/v) glucose medium being at a minimum when the culture pH value is 5.4. The activity increases as the pH value is reduced to 3.5 or increased to 7.7. Between pH 7.7 and 8.3 the activity decreases again, probably due to an increase in the glucose concentration in the culture over this range. The optimal effective activity is obtained with a culture at pH 3.5. Since the invertase

activity of organisms grown in a 2% (w/v) glucose medium over a wide range of pH values is low, it would appear that the inhibitory effect of high concentrations of glucose occurs at all pH values.

In the introduction it was stated that by use of the continuous culture technique it should be possible to assess the part played by changes in the rate of division and in the concentration of various nutrients on the invertase content of the organisms. It has now been shown that the rate of division and the concentration of ammonium ions and growth factors in the culture have no significant effect on the invertase content of the organisms under the conditions employed. As the pH values of the cultures (2.6–3.4, depending on sugar concentration) normally shows little change, this factor also has little effect on the invertase content, though, as described above, large changes in pH value have a marked effect. The main factor which controls the invertase content of *Saccharomyces fragilis* in media containing glucose is the glucose concentration.

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A Survey of Physically Active Organic Infusoricidal Compounds and their Soluble Derivatives with Special Reference to their Action on the Rumen Microbial System

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SUMMARY: Many sparingly water-soluble neutral substances, including several indole derivatives, will quickly kill and often disintegrate washed living rumen ciliates at 35–39°, when acting at or near the saturation point (0.002–0.1 M) in a phosphate + acetate buffer at pH 7. The toxic compounds are mostly readily soluble in light petroleum. Organisms of the genera *Isotricha*, *Dasytricha* and *Ophryoscolex*, in particular, exhibit characteristic modes of disintegration when acted upon by such substances. Prominent among these are the terpene alcohols menthol, borneol and isoborneol, and their more soluble glucosides (both α - and β -) and their acid succinates. Menthoxycetic acid is also similarly toxic. On the other hand, menthol and borneol β -glucuronides are not toxic to the rumen ciliates unless added to rumen contents containing bacteria also. Unlike menthol, borneol, in nearly saturated solution, is not particularly toxic to the rumen bacterial species *Streptococcus bovis* and *Sarcina bakeri* and quite high concentrations (0.1–0.4%) of the terpenoid succinates and glucosides are required for even partial inhibition of the growth of true rumen saccharolytic bacteria under otherwise favourable conditions. The population of lipolytic (esterase producing) bacteria in rumen contents from a hay and dried grass-fed sheep was of the order of 500,000/g. rumen contents. *Bacillus licheniformis* was the chief anaerobic lipolytic species isolated.

Although much is now known about carbohydrate fermentations by rumen ciliate protozoa (see Hungate, 1955, for summary), very little success has attended efforts to cultivate individual species in chemically defined media. Consequently, their nitrogen metabolism is still a closed book, and we do not know whether the conversion of part of the fodder organic-N into protozoan protein of higher nutritional value, which undoubtedly takes place in the rumen (see McNaught, Owen, Henry & Kon, 1954), is accompanied by an appreciable loss of fodder protein nitrogen as ammonia, or by destruction of B-vitamins synthesized by rumen bacteria. To obtain evidence on these points it might help greatly to be able to maintain individual protozoan species in the rumen of living sheep. The simplest way to do this might be to defaunate a sheep under conditions bringing about the minimum of harm to the rumen bacteria and to the host and then to introduce the single ciliate species under investigation in the hope that it would multiply and maintain itself in the rumen. Something along these lines has already been attempted by Becker, Schulz & Emmerson (1930) with goats, but they used a relatively drastic method of defaunation, viz. long-continued starvation followed by dosing with copper sulphate solution at definitely bactericidal concentrations. New infusoricidal agents, which have little toxic action on the host or its rumen bacteria,

seem to be required for the end in view. This paper explores the possibilities of physically active organic substances (cf. Ferguson, 1939) in this respect, by means of *in vitro* methods.

Eadie & Oxford (1954) showed that the sparingly soluble neutral substances indole and skatole, which might even arise naturally by bacterial action on tryptophan in the rumen, were very toxic to rumen ciliates when acting at or near the aqueous saturation point. We have now shown that this is a general property of sparingly water-soluble but readily hydrocarbon-soluble, neutral substances, and have particularly directed our attention to the terpene alcohols since these can be made more water-soluble by linking to residues, such as glucose, glucuronic or succinic acids which are known to be attacked by rumen bacteria. If these compounds are so attacked in the rumen itself, a saturated solution of the terpene alcohol might quickly be produced throughout its contents following one massive dose of the solubilized form of the alcohol. The advantages of borneol and isoborneol in this respect are increased by the ease with which mammals can excrete such compounds in the urine, almost exclusively as β -glucuronides (see Williams, 1947). Rumen bacteria are known to secrete α -glucosidase (e.g. *Streptococcus bovis* ferments maltose), β -glucosidase (Conchie, 1954), β -glucuronidase (Karunairatnam & Levvy, 1951), and an enzyme system fermenting succinate (see Sijpesteijn & Elsdon, 1952), although no one seems previously to have studied rumen bacterial esterase such as might hydrolyse the alkyl esters of succinic acid to the free alcohol and acid. We have therefore completed our study by computing and isolating the chief lipase (esterase)-containing bacteria, in the rumen of a hay and dried grass-fed sheep, which are able to act anaerobically.

METHODS

Compounds tested for infusoricidal activity

Unless otherwise stated, the compounds listed below were good-quality laboratory reagents purchased from British Drug Houses Ltd. or L. Light and Co. Ltd. Other compounds were specially prepared or given by the persons mentioned in the acknowledgements at the end of this paper.

(a) *Hydrocarbons*: toluene (Analar, redistilled); naphthalene.

(b) *Non-nitrogenous alcohols*: borneol; dulcitol; geraniol; isoborneol; menthol; 1-phenylethanol (Klages & Allendorff; 1898); 2-phenylethanol; stilboestrol; terpin hydrate; β -ionone (an odoriferous ketone) was also included for comparison.

(c) *Non-nitrogenous ethers*: anisole; cineole; menthoxyacetic acid (*Organic Syntheses*, 1955).

(d) *Indole derivatives*: di-indolylmethane; ω -hydroxyskatole (Leete & Marion, 1953); 1-methylindole (Potts & Saxton, 1954); 7-methoxy-2-methylindole (see acknowledgements); 4-methoxy-1-methylindole; 5-methoxyindole; 5-methoxy-1-methylindole; 7-methoxy-1-methylindole; 7-methoxy-1:3-dimethylindole (see acknowledgements); 2-phenylindole; skatole (recrystallized from light petroleum); tryptophol (2-indolylethanol; Ehrlich, 1912).

(e) *Glucosides and glucuronides with sparingly soluble aglycones*: menthol- β -glucoside (Fischer & Raske, 1909); borneol- α -glucoside, menthol- β -glucuronide (see acknowledgements). Borneol- β -glucuronide. This was prepared as follows: borneol (5 g. in aqueous suspension) was administered directly through the cannula to the rumen of a fistulated hay-fed sheep, on six separate occasions more or less evenly spread over 3 days. During this period, its urine was collected, pooled, and from it was prepared the insoluble zinc salt of borneol- β -glucuronide as described by Quick (1927) for borneol-fed dog urine. Since the preparation of the free glucuronide from the zinc salt is said not to be quantitative, it was considered best, for the purposes of *in vitro* testing, to convert the crude zinc salt into the soluble potassium salt by grinding the dried material (3 g.) with a solution of K_2HPO_4 (2.1 g.) in distilled water (75 ml.). After standing for 1 hr. with occasional stirring, the insoluble zinc phosphate was removed by centrifuging and the supernatant finally cleared by filtration to yield an approximately 1.3% (w/v) solution of borneol- β -glucuronide at pH 7.

(f) *Monoalkyl succinates yielding sparingly soluble alcohols on hydrolysis*. Monomenthyl, monobornyl, and mono*isobornyl* succinates were prepared by heating the appropriate terpene alcohol with excess of succinic anhydride at 150° (not higher because of possible dehydration of the alcohol to yield hydrocarbons) for 6 hr. (Beckmann, 1909). The melt was cooled, triturated with cold light petroleum (b.p. 40–60°; in which succinic anhydride is insoluble) filtered and the filtrate decolorized with a little charcoal, and finally evaporated to dryness *in vacuo* (20 mm.) at 50°. Crude monomenthyl succinate so prepared was a solid m.p. 50° (lit. 59°) and was purified by recrystallization from aqueous ethanol. The other two acid succinates, although of approximately correct equivalent weights, were pale yellow oils. Since the starting products were not optically pure it was not considered worth while to attempt to purify these esters. All three monoalkyl succinates were completely soluble in cold sodium carbonate solution.

(g) *Compounds acting entirely, or in part, by chemical toxicity*. For comparative purposes, the very sparingly soluble phenolic substance eugenol and the organic arsenical, *m*-amino-*p*-hydroxyphenyl-arsine hydrochloride (Mapharside; Parke, Davis and Co.) were also tested. The first-named compound no doubt can partly act by a physical mechanism also. It was included because it is the aglycone of the naturally occurring plant β -glucoside gein.

Preparation of washed suspensions of living rumen ciliate protozoa for testing the infusoricidal compounds. The method of Eadie & Oxford (1955) for obtaining washed suspensions of undamaged motile ciliates was used, preferably with addition of galactose rather than glucose. Some living oligotrich ciliates were always present in these suspensions. The same two fistulated hay-, or hay and dried grass-fed sheep were employed as in the previous study, viz. nos. 1004 and 8060. The latter animal was especially useful for providing oligotrichs including *Ophryoscolex* spp. The buffer solution used for removal of extraneous bacteria, by washing by decantation, and for subsequent maintenance of the protozoa in the living state, was again the simplest possible, viz. (% w/v)

NaCl, 0.5; anhydrous sodium acetate, 0.13; KH_2PO_4 , 0.03; K_2HPO_4 , 0.10; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; pH 7.2. During the first few hours in this buffer at 39°, the oligotrichs, as well as the holotrichs, remained alive, i.e. with visible ciliary motion even when the organism as a whole remained stationary; so that a toxic effect of an added substance on all the rumen genera could be demonstrated when the test was carried out immediately after preparation of the washed protozoan suspension. When the washed protozoan deposit at the bottom of a 50 ml. boiling tube filled with buffer was allowed to stand overnight at 39°, no especial precautions being taken to exclude air or to maintain highly reducing conditions, only the holotrichs and *Ophryoscolex* survived. Such suspensions could therefore be used for confirming results obtained on the previous day, with respect to three rumen genera only. Small preparations of living *Isotricha* free from *Dasytricha*, and of *Dasytricha* free from *Isotricha*, were obtained by the method of Gutierrez (1955).

Methods for testing infusoricidal activity against washed suspensions of ciliates. The solubility of a neutral compound under test in the buffer at about 45° was first roughly determined by trial and error. Slightly more of the compound than necessary to give 10 ml. saturated solution at this temperature was then weighed into a 5 × 0.5 in. (internal diam.) test-tube. Buffer solution (10 ml.) was added to fill the test-tube about two-thirds full. The tube was placed in a water-bath at 45° and its contents slowly stirred by hand, by means of a thin glass rod, for at least 15 min. until no more substance appeared to dissolve. When an appreciable amount of the substance appeared to remain undissolved, the liquid was filtered through a very small fluted Whatman no. 1 paper into another test-tube. Skatole (4 mg./10 ml.) was always used in the positive control tube and a no-substrate control (i.e. buffer only) was also always included. The water-bath (usually a 250 ml. beaker) containing the test-tubes immersed up to the level of the liquid inside them, was then placed in a 39° incubator, and when the temperature of the water in the bath had fallen to 38°, 0.1 ml. of clean washed ciliate suspension was added to each tube. This was best achieved in the following way: the washed stock ciliate suspension in buffer at the bottom of a 50 ml. boiling tube, and a number of graduated 1 and 2 ml. pipettes, slides and coverslips having been placed in the incubator well beforehand, a pipette-full of the bottom protozoan layer was quickly sucked up when required for inocula and 0.15 ml. immediately run out into each of the test-tubes in the water-bath. Great expedition was here required, otherwise the protozoa would settle under gravity, even in the pipette itself. Each inoculated tube was rotated by hand in such a way that the protozoa were evenly suspended without the introduction of air bubbles into the liquid. In some instances, dilutions in buffer were made from a saturated solution and inoculated likewise in order to determine whether a nearly saturated solution was needed for the full toxic effect. The appearance of the protozoa was noted after 30, 60, 120, and 180 min. by the following procedure. The tube was first shaken as before to indicate whether a permanent turbidity (due to release of storage grains and inclusions from disrupted organisms) was produced. Five min. later, about 0.02 ml. of the bottom deposit was quickly transferred to

a slide, the cover-slip added and the preparation quickly transferred to a not too cold microscope stage. The microscope itself could be advantageously kept in a 39° incubator between periods of use.

A convenient source of illumination was northern daylight and the $\frac{3}{8}$ -inch objective would reveal sufficient detail for the purpose. Besides obviously disrupted and obviously motile ciliates, those motionless as a whole were carefully examined to discover whether all ciliary motion had entirely ceased. The temperature of the water-bath in the incubator was at no time allowed to fall below 35°. When necessary it was from time to time taken out and warmed to 39° on a wire gauze over a small bunsen burner.

When the compound to be tested was acidic, an endeavour was made to dissolve it directly in the buffer. Provided the pH value did not fall below 6.5, the test could then be carried out. Otherwise, appropriate volumes of a concentrated solution of the acid in the correct amount of NaOH solution for neutralization were added to at least 10 vol. of the buffer to give the concentrations of toxic agent required.

Methods for testing infusoricidal activity against the ciliates in the rumen microbial system as a whole containing bacteria also. Strained rumen liquor without added sugar (50 ml.) was placed in each of two pear-shaped 100 ml. separating funnels kept in the incubator. To one was added the toxic agent in neutral buffer solution, the total volume in the funnel being made up to 75 ml., when necessary, with buffer. To the other was added buffer solution only (25 ml.). The contents of the funnels were mixed after 0, 15, 30 min. and so on, as necessary. Five min. later a little of the bottom deposit in each was withdrawn as before and examined under the microscope. When complete mortality of the ciliates seemed to have occurred in the funnel containing toxic agent, 1 ml. of washed living ciliate suspension was added to each funnel and the incubation continued as before, with frequent microscopic examination of the bottom deposits.

Bactericidal effect of infusoricidal compounds upon authentic rumen bacteria

Isolation of Streptococcus bovis after action of the compound upon the whole rumen microbial system. *Streptococcus bovis*, the typical rumen starch-fermenting streptococcus (MacPherson, 1953), is not usually found as an air or fodder-borne contaminant (Mann, Masson & Oxford, 1954). Its isolation from the rumen microbial system after death of all rumen ciliates is therefore at least an indication that not all true rumen bacteria have been killed by the infusoricidal agent. This isolation was attempted by transferring loopfuls of well-shaken funnel contents (see previous section) into tubes of glucose + Lemco broth, and after 18 hr. of incubation at 38°, when usually a dense turbidity had developed and the pH value had fallen to 4–5, the culture was examined for Gram-positive cocci existing in pairs and chains. When these were observed, streaks were made on to starch nutrient agar plates. After overnight incubation the plates were flooded with dilute Lugol's iodine in order to detect clear zones of starch hydrolysis around any tiny typically strepto-

coccal colonies which might have developed. These had to be searched for, since the preponderant growth was always of Gram-positive rods.

Inhibitory effect of soluble infusoricidal agents upon the anaerobic development, in nutrient agar, of rumen bacteria taken directly from the rumen. This was attempted only with the glucosides and acid succinates of the terpene alcohols. An approximately 2% (when feasible, otherwise an almost saturated) solution of the compound was adjusted to pH 7-8 and sterilized by filtration through a Berkefeld candle (type kN; Baird & Tatlock Ltd.,) and the filtrate incorporated into a number of plates of nutrient agar, prepared as described by Huhtanen, Rogers & Gall (1952) to give a range of concentrations from 0.5 to 0.01% in the final agar. The inoculum for each poured plate (including one containing no toxic agent) was 1 ml. of a 10^{-4} dilution of rumen contents freshly taken from sheep 8060. Colonies were counted after 4 days of anaerobic incubation at 38°.

Bactericidal action of menthol, borneol and 2-phenylethanol upon pure cultures of true rumen bacteria. *Streptococcus bovis* and *Sarcina bakeri* (Mann *et al.* 1954) were the test organisms. Saturated aqueous solutions of the compounds were sterilized by filtration through sintered glass (Jobling Pyrex; bacteriological grade 5/3), and added to triple-strength glucose nutrient broth to give a maximum final concentration of two-thirds saturation. All tubes were made up to the same volume with sterile distilled water and inoculated with a loopful of a young broth culture of the test organism. Tubes were examined after 2 days of incubation at 38° and in order to confirm an apparent bactericidal effect subcultures were made from those which showed no turbidity. Further tests, with appropriate solvent and medium controls, were also made by adding sterile 11.2% (w/v) ethanolic solutions of the compounds to glucose nutrient broth, up to a maximum of 1.3% (v/v) ethanol in the final medium.

Isolation of lipase-producing rumen bacteria in pure culture. The assumption being made that bacteria capable of hydrolyzing tributyrin at pH 6-7 might also hydrolyse at this pH value the monoalkyl succinates used as potential infusoricidal agents, it became of interest to determine whether the rumen mixed culture really contained an appreciable population of such bacteria acting anaerobically. The Huhtanen, Rogers & Gall (1952) technique previously employed (see above) was therefore modified by incorporation of 1.25% (v/v) tributyrin (British Drug Houses Ltd.), sterilized separately in concentrated gelatin suspension (Eisenberg, 1939), into the nutrient agar to give a well-marked turbidity. Serial dilutions of freshly withdrawn rumen contents were employed as inocula for poured plates as before. After 4 days of anaerobic incubation at 38° the number of colonies in each plate surrounded by a definite zone of clearing was counted. Subcultures were made from such colonies to fresh tributyrin agar and if zones of clearing again appeared upon anaerobic incubation, the isolates were purified and attempts made to identify them by the standard procedures for the genus in question.

RESULTS

Nature of the visible damage sustained by rumen ciliates due to physically-active compounds

As stated by Eadie & Oxford (1954) the outer membranes (ectoplasmic zone) of the holotrichs, to which the cilia are attached, were broken or torn off, sometimes indeed apparently 'dissolved' away with liberation of storage and other granules from the endoplasm, which often streamed away from the wrecked organism. *Ophryoscolex* also exhibited a characteristically damaged appearance, resembling a thick half-snapped twig; and the relatively lengthy dorsal membranelle zone of cilia, which is clearly the chief region of mechanical weakness in the organism, was often completely ripped off, with consequent oozing out of the internal contents. The other oligotrichs (*Diplodinium* and related genera and *Entodinium*) have however a tough outer cuticle (Dogiel, 1927) which protects the ciliary zones. Consequently, they showed no obvious damage save the cessation of ciliary motion. By contrast, the chemically active Mapharside, although lethal at a low concentration (0.005%) did not cause disintegration even at much higher concentrations, the holotrichs for example dying with their ectoplasmic zone intact and their cilia extended more or less rigidly. The dorsal membranelle zone of *Ophryoscolex* was also unaffected by Mapharside.

Solubilities in water and infusoricidal action at and below saturation point of neutral compounds tested

The literature contains few determinations of aqueous solubility of these compounds at 40° or thereabouts. Table 1 lists our own rough solubility determinations and such information as we could find in the literature for 'room temperatures'. It also indicates whether or not the compound, in nearly saturated solution, is characteristically toxic. The following practically insoluble compounds, not included in Table 1, were not highly toxic: di-indolyl-methane; 4- and 5-methoxy-1-methylindoles; 7-methoxy-1:3-dimethylindole; 2-phenylindole; ω -hydroxyskatole was too rapidly decomposed by warm water to give reliable results by the method used. Of the compounds which are appreciably water-soluble and are listed in Table 1 as not particularly toxic, dulcitol is insoluble, terpin hydrate very sparingly soluble and anisole readily soluble in light petroleum (b.p. 40–60°) at the boiling point. All the toxic compounds, with saturation points at 0.002–0.1 M are appreciably soluble in light petroleum with the exception of the glucosides, which are insoluble.

Irrespective of actual solubility at the saturation point, the toxicities of the purely physically-active compounds in Table 1 fell off quite rapidly with decreasing concentration, so that at concentrations less than half saturation no particular action on the ciliates was shown over long periods of time. Exceptions to this behaviour were provided by eugenol, 2-phenylethanol and the glucosides. These compounds were fully active at concentrations well below half saturation. Menthol- β -glucoside (0.2%) was equally toxic to *Isoetricha* and *Dasytricha* both in mixed and in separate 'pure' suspensions.

Action of the acidic glucuronides, succinates and menthoxyacetic acid on washed suspensions of rumen ciliates

The glucuronides were quite inert. Menthol- β -glucuronide, for example, even at a concentration of 0.025 M, in buffer to which enough NaHCO_3 had been added to bring to pH 7.2, had no action on the holotrichs during several hours at 38°.

Table 1. *Solubilities of sparingly soluble neutral compounds in water at temperatures up to 45° and action on washed ciliates at or near saturation point at 35–39°*

Compounds	Solubility at 45° unless otherwise stated (mole/l.)	Action on ciliates (++ = death and disintegration + = death only 0 = not highly toxic)
Anisole	0.01	0
Borneol	0.004, 0.005 (25°)*	++
Borneol- α -glucoside	0.01 (or greater)	++
iso-Borneol	0.002	++ (if unfiltered)
Cineole	0.02, 0.01 (20°)*	++
Dulcitol	0.2 (16°)*	0 (no action in 18 hr.)
Eugenol	0.006	++
Geraniol	0.004	++
β -Ionone	0.003	++
Menthol	0.004, 0.0025 (20°)*	++
Menthol- β -glucoside	0.013	++
1-Methylindole	0.003	++
5-Methoxyindole	0.005	+
7-Methoxy-1-methylindole	0.005	++
7-Methoxy-2-methylindole	0.0025	++
Naphthalene	0.0002 (20°)*†	0 (but definite deleterious action on holotrichs)
1-Phenylethanol	0.02	++
2-Phenylethanol	0.08, 0.13 (20°)*	++
Skatole	0.0025, 0.003 (20°)*	++
Stilboestrol	< 0.001	++
Terpin hydrate	0.02 (20°)*	0
Toluene	0.005 (16°)*, 0.007 (25°)†	++
Tryptophol (2-indolyethanol)	0.045	++

* Handbook (1947).

† Weissberger & Proskauer (1955).

The monomenthyl, monobornyl, or mono*isobornyl* esters of succinic acid, when dissolved directly in the buffer at a concentration of *c.* 0.15% (0.006 M; final pH 6.5) were fully toxic against all rumen ciliates in 1–2 hr. at 35–39°. Menthoxyacetic acid, which like the above acid esters, is readily soluble in the free state in light petroleum, was similarly toxic at a rather higher concentration (0.015 M).

Action of acidic glucuronides, succinates and menthoxyacetic acid on the ciliates in whole rumen liquor containing bacteria

The compounds mentioned in the last paragraph were again quickly lethal to the ciliates as was also menthol- β -glucoside, but in the first three instances and the glucoside the smell of the terpene alcohol itself was in evidence within 1 hr. This indicates that rapid bacterial hydrolysis of the succinates and also of menthol- β -glucoside had taken place. The β -glucuronides of menthol and borneol, however, required at least 24 hr. of incubation at 39° to bring about the same effect. Within 6 hr., there was little action on the rumen ciliates and the smell of the terpene alcohol was not perceptible. In all the above instances the presence of viable *Streptococcus bovis* was demonstrated without difficulty after death of the ciliates. In the experiment with monobornyl succinate, a million-fold dilution of the rumen liquor at the end still contained this bacterium in the viable state.

Inhibitory effect of soluble glucosides and succinates in nutrient agar upon the development of bacteria freshly taken from the rumen. By the technique used, the colony count in the nutrient agar without infusoricidal agent was always of the order of 1000–2000 colonies/plate. The smallest concentrations of infusoricidal agents required to reduce this count by *c.* 90 % (i.e. to *c.* 100–200 colonies/plate) were as follows: menthol- β -glucoside, 0.25 %, monomenthyl succinate, 0.1 %, monobornyl succinate, 0.4 %, monoisobornyl succinate 0.4 %. Mapharside was so toxic that no colonies of rumen bacteria developed even when the concentration of the arsenical in the nutrient agar was as little as 0.005 %.

Antibacterial action of menthol and borneol upon Streptococcus bovis and Sarcina bakeri. In liquid media without ethanol, 67 % saturation with menthol or borneol had no apparent effect on the growth of either organism. 2-Phenyl-ethanol similarly used was, however, bactericidal to both. In media containing 1.3 % (v/v) ethanol, a saturated solution of menthol, but not of borneol, was bactericidal towards *Streptococcus bovis*. The results with *Sarcina bakeri* were vitiated by the fact that ethanol itself, at the concentration used, was bactericidal to the organism. As far as could be judged with liquid media containing much less ethanol (0.33 %, v/v) a saturated solution of borneol had little effect on the growth of this organism. A corresponding aqueous ethanolic solution of menthol was however definitely bactericidal. In general, menthol seemed always to be more bactericidal than borneol.

Density of population and nature of lipase-producing bacteria on rumen contents

The highest dilutions of rumen contents from sheep 8060, fed on hay and dried grass, which when incorporated in tributyrin agar definitely gave rise to several colonies with zones of clearing around them, were always 10^{-4} and 10^{-5} . A population of *c.* 500,000 lipase-producing bacteria/g. rumen contents was therefore indicated. The zones although very distinct were always narrow, with only 1–2 mm. between the edge of the colony and the limit of clearing. Nearly

all the organisms isolated from these colonies proved to belong to the genus *Bacillus*, and most of the isolates when examined by the procedures of Appleby (1955) proved to be *B. licheniformis*. In fact, several of the strongly proteolytic *B. licheniformis* rumen isolates obtained by Appleby (1955) proved to be lipolytic also when grown anaerobically in tributyrin agar. The lipase of *B. licheniformis* seemed to be a perfectly stable constituent of this species, still retained after many subcultures on ordinary fat-free media. Other well-known rumen organisms, such as *Streptococcus bovis* although growing anaerobically on tributyrin agar produced no zones of clearing around the colonies.

DISCUSSION

Since it is unlikely that the rumen ciliates possess an enzyme capable of hydrolysing the ether link in a stable compound like menthoxyacetic acid (cf. the non-toxicity of phenylmethyl ether to these organisms), it may be supposed that, by analogy, the acid succinates of the terpene alcohols are also toxic in themselves. It is significant that, at an acidic pH value but not at pH 7, all these acids are readily soluble in light petroleum, and therefore presumably in lipid materials in general, just as the free terpene alcohols are. There is no need to postulate a ciliate esterase in order to account for the toxicity of the monoalkyl succinates, provided it be assumed that the internal contents of the ciliates are definitely acidic in reaction. This may well be so since Heald & Oxford (1953) and Gutierrez (1955) showed that lactic acid is one of the chief fermentation products, both of glucose and endogenously of storage polysaccharide, by the holotrichs at least. Since rumen bacterial lipase (esterase) undoubtedly exists, these terpene succinates may, in the rumen, be toxic to the ciliates twice over, both before and after hydrolysis, whereas the terpene glucosides have probably only one mode of toxic action, namely after hydrolysis by protozoan or bacterial enzymes.

Incidentally, although indole and skatole are toxic to the ciliates, indoleacetic, -propionic, and -butyric acids unlike menthoxyacetic acid are not (see Eadie & Oxford, 1954). This may be due to the complete insolubility of these indole derivatives in lipid materials; they are certainly insoluble in light petroleum. The non-toxicity of dulcitol and terpin hydrate may be likewise explained.

One anomaly still remains. Gutierrez (1955) produced good evidence in favour of the view that only *Dasytricha* among the holotrichs contained a β -glucosidase enzyme system, whereas we find that menthol- β -glucoside is indifferently toxic to all three species of rumen holotrichs, even when *Isotricha* is separated free from *Dasytricha*, or vice versa. The possibility that washed suspensions of *Isotricha* contain large numbers of bacteria with a β -glucosidase enzyme system inside the ciliates, is considered unlikely in view of the failure of Sugden & Oxford (1952) to demonstrate any such bacteria. The question is being further studied, use being made of more specific staining methods. The inertness of the terpene glucuronides is in agreement with the known absence of a ciliate β -glucuronidase (Karunairatnam & Levvy, 1951). It is clear also

from our results that the rumen bacterial β -glucuronidase is far less potent than the β -glucosidase possessed by the same mixed bacterial culture.

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The Production of Capsules, Hyaluronic Acid and Hyaluronidase by Group A and Group C Streptococci

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SUMMARY: Some capsulated group A and group C streptococci were found to produce both hyaluronic acid and hyaluronidase during active growth; thus confirming and extending the work of Pike (1948*b*). In a group C strain, LM, the free hyaluronic acid in liquid culture represents an excess of synthesis over destruction, the balance being influenced by the aeration of the culture. Within the limits examined the degree of aeration did not, however, influence hyaluronidase or hyaluronic acid production by strains producing either the enzyme or its substrate exclusively. A non-capsulated, hyaluronidase producing mutant of strain LM was isolated but capsulated cells, free from this variant, also produced the enzyme. Evidence is presented that the spreading activity of strain LM preparations in rabbit dermis is due to hyaluronidase rather than to capsular material as suggested by Pradhan (1937).

McClean (1941) showed that some strains of streptococci develop capsules in young cultures, whereas others produce hyaluronidase and are not capsulated. Further studies (Crowley, 1944; Pike, 1948*a*; Russell & Sherwood, 1949; Murray & Pearce, 1949) in which a variety of hyaluronidase assay methods were used appeared to establish the fact that streptococci can be classified into strains producing both capsules and hyaluronic acid, those producing hyaluronidase and those producing neither the enzyme nor the acid. Pike (1948*b*), however, found that seven of ten capsulated, hyaluronic acid producing, group A strains form a filterable, thermolabile agent which destroys the hyaluronic acid in ageing cultures. More recently, Faber & Rosendal (1954) have described a fall in hyaluronic acid concentration in ageing cultures of 35 of 47 group A strains that produce the polysaccharide, the other 12 strains showing no fall in concentration. There is little direct evidence that the slow destruction of hyaluronic acid by cultures of some group A strains is due to a hyaluronidase beyond the fact that the destructive agent is thermolabile and filterable. It is justifiable, however, to refer here to the destructive agent as hyaluronidase since in another paper (MacLennan, 1956) the isolation and characterization of an enzyme resembling other streptococcal hyaluronidases from cultures of a hyaluronic acid-producing group C streptococcus is described. The object of the present work was to examine in more detail the relationships of capsulation, hyaluronic acid and hyaluronidase production in some group A and group C streptococci at all stages of growth.

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MATERIALS AND METHODS

Source of streptococci. Two capsulated, hyaluronic acid-producing, group A strains, A 118 (Griffith's type 19) and A 111 (Griffith's type 18), were gifts from Dr R. Pike who reported (Pike, 1948*b*) that A 118 but not A 111 produced hyaluronidase. A third group A strain, type 1 (NCTC 8198) was also examined. The three group C strains were LG (NCTC 6178), C 7 (NCTC 4540), known to be a hyaluronidase producer, and strain LM (NCTC 6176), which was described by Pradhan (1937) as having spreading factor activity in young serum broth cultures.

Cultivation of streptococci. Cultures were grown in a 2.5 % Evans' peptone, 10 % horse serum, 1 % glucose, horse meat infusion broth, hereafter PSB, from inocula of once-washed saline suspensions of overnight growth in the same medium. Each 100 ml. of medium was seeded with organisms from 10 ml. of overnight growth in the same medium suspended in 1 ml. saline. Cultures were kept at pH 7.0-7.6 during growth by the addition of 10 % sodium hydroxide. A replicate culture containing 0.002 % phenol red was used as a guide to neutralization since the presence of phenol red in experimental cultures led to high blank values in the colorimetric estimation of hyaluronic acid.

For aerobic and anaerobic cultivation respectively, oxygen or nitrogen gas, each containing 5 % carbon dioxide, was bubbled into cultures through perforated rubber tube spargers. Silicone Antifoam A was added to prevent foaming.

Measurement of growth. Bacterial N was measured on washed cells by the micro-Kjeldahl method.

Capsulation. A loopful of culture and a loopful of cobalt blue ink (Uno waterproof ink) were mixed on a grease-free slide, smeared and then dried without heating. After staining with 1/6 Löffler's methylene blue for 3 min. and washing in water the preparation was dried without heating. Dr Nuala Crowley (personal communication) suggested the use of 'Uno' ink which gives a more uniform background than other inks.

Hyaluronic acid. The turbidimetric method described by Pike (1946) was used with Allen and Hanburys hyaluronic acid as a standard. Turbidities were measured on the Evans' Electroselenium Co. Ltd. (EEL) colorimeter using a blue filter, No. 622.

Hyaluronidase. This was estimated by the mucin clot prevention (M.C.P.) test as described by McClean (1943). For the test hyaluronic acid of high viscosity was prepared from human umbilical cords by the method of McClean (1943). To detect small quantities of hyaluronidase the incubation period of the test was increased from 20 min. to 24 hr. and thiomersalate was added to the enzyme substrate reaction mixture to a concentration of 1/10,000.

Small amounts of hyaluronidase in culture supernatants were also detected by a method differing only in detail from that of Pike (1948*a*). Two ml. of culture supernatant and 2 ml. of a solution of 40 mg. hyaluronic acid (Allen and Hanburys)/100 ml. culture medium were mixed and incubated at 37° for a period which was varied according to the activity of the enzyme present,

but which was of course the same for any experiment in which activities were being compared. The mixture was held at pH 7.3-7.5 and contained 1/10,000 thiomersalate. The hyaluronic acid remaining after incubation was determined turbidimetrically by Pike's method (1946). Enzyme activity was expressed as mg. hyaluronic acid destroyed/100 ml. of reaction mixture.

RESULTS

A survey of strains

Figure 1 records the growth and hyaluronic acid production of 3 group A and 3 group C strains after one passage through mice. Hyaluronidase production and capsulation were also measured at hourly intervals. In order to detect small amounts of hyaluronidase the incubation period of the M.C.P. test was

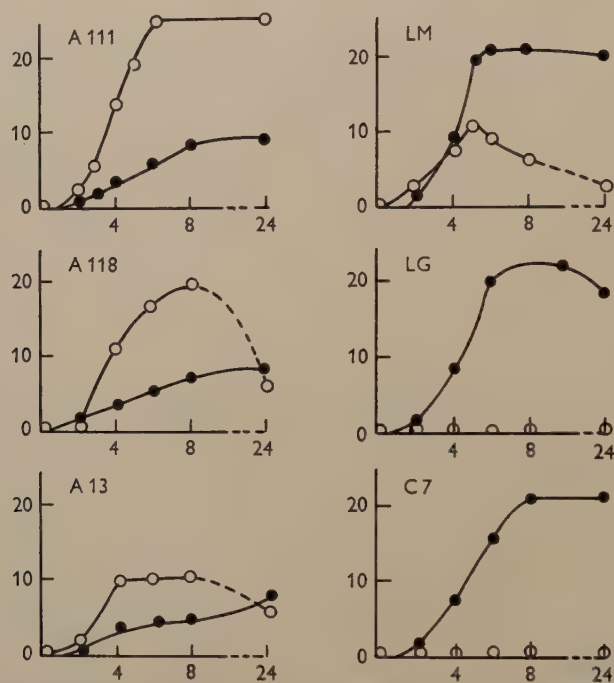


Fig. 1. The growth and hyaluronic acid production of streptococci. Ordinates: mg. hyaluronic acid/100 ml. culture (○—○) and mg. bact. N/100 ml. culture (●—●). Abscissae: age of culture (hr.).

increased to 24 hr. Group C strains grew more regularly and more abundantly than did group A strains. Two of the group C strains, LG and C7, were without capsules at all stages of growth and failed to produce hyaluronic acid; LG produced no detectable hyaluronidase, but strain C7 produced very high titres throughout growth.

Strain LM and the three group A strains, on the other hand, produced capsules and hyaluronic acid. Production was associated with the phase of

active growth as reported by Seastone (1939), Morison (1940) and others. In all four strains the capsules were lost towards the close of the log phase and in three, A 13, A 118 and LM, there was a corresponding fall in the concentration of hyaluronic acid in the cultures. This fall in ageing cultures of A 13, A 118 and LM and the persistence of the polysaccharide in those of A 111 confirms and extends the observations of Pike (1948*b*). Strain LM is the first group C strain to have been shown to produce both hyaluronic acid and hyaluronidase. Since this work was begun Faber & Rosendal (1954) have reported hyaluronidase formation by hyaluronic acid producing strains of the three group A serological types 1, 19 and 18 represented here by A 13, A 118 and A 111 respectively.

Because the M.C.P. test had failed to detect hyaluronidase in those cultures in which hyaluronic acid had been destroyed it seemed probable that the failure of earlier workers (McClean, 1941; Crowley, 1944) to demonstrate enzyme activity in cultures of capsulated strains by the M.C.P. test using a much shorter incubation period was due to its relative insensitivity. To verify this a comparison of the M.C.P. test (20 min. incubation) and the turbidimetric method (16 hr. incubation) described above was made. The enzyme activity of a strain LM culture supernatant free from hyaluronic acid was compared turbidimetrically with that of a series of dilutions of the supernatant of the hyaluronidase producing strain C 7 containing 1 M.C.P. unit/ml. and less. The destruction of hyaluronic acid by C 7 dilutions containing only 0.04–0.2 M.C.P. units was equal to that of the undiluted LM supernatant.

Strain LM differed from strain A 111 in the small amount of hyaluronic acid present despite good capsulation. It was thought that this might be due to a rapid enzymic breakdown of hyaluronic acid liberated from the capsule by this strain. Duplicate cultures of strain LM were therefore sampled at intervals during growth. The samples were centrifuged and each supernatant sterilized by 1/10,000 thiomersalate, duplicate portions being then stored for 16 hr. at 2° and 37° respectively. Residual hyaluronic acid was then determined. The complete disappearance of hyaluronic acid from all supernatants stored at 37° shows clearly that hyaluronidase is liberated into the culture medium at an early stage of growth (Table 1). This observation was consistently reproduced; no culture supernatant of strain LM was ever found to contain hyaluronic acid that was not destroyed by incubation at 37° after sterilization. The curve in Fig. 2 represents the polysaccharide content of a growing strain LM culture and the three curves arising from this show the fall in concentration that occurred in 4¼, 6 and 7½ hr. sterilized samples of culture supernatants when incubated at 37°. During growth between 4¼ and 6 hr. the concentration of hyaluronic acid rose by 7 mg./100 ml. and yet if growth were prevented during this period there was a fall of 2.5 mg./100 ml. Moreover, in the growing culture the hyaluronidase was acting on a constantly increasing concentration of polysaccharide, whereas under bacteriostatic conditions the substrate concentration fell steadily. From what is known of enzyme kinetics it is likely that the loss of hyaluronic acid during 1¾ hr. growth was greater than 2.5 mg./100 ml. The results of these two experiments show clearly that the

amount of hyaluronic acid found in a strain LM culture represents a balance of synthesis and destruction. As will be shown later the presence of the enzyme in young cultures of strain LM is of interest in the light of Pradhan's (1937) report that young cultures of this strain in the capsulated phase had spreading factor activity in rabbit dermis; he attributed this activity to the capsular substance itself.

Table 1. *The time of appearance of hyaluronidase in cultures of strain LM*

Age of culture (hr.)	Mg. hyaluronic acid/100 ml. of culture. Supernatants stored at			
	2°		37°	
	1	2	1	2
2	0	0	0	0
4	5.5	4.3	0	0
5	10	8.7	0	0
6	6.3	5.2	0	0
7	6.2	5.2	0	0
8	5.7	—	0	0
24	0	0	0	0

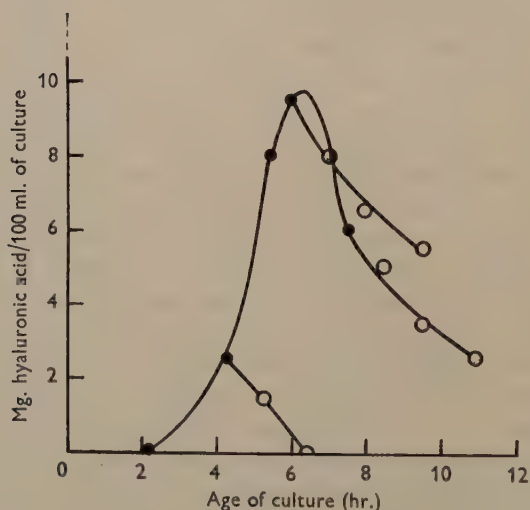


Fig. 2. The rates of accumulation and destruction of hyaluronic acid in a culture of strain LM. Accumulation during growth (●—●); destruction in the absence of growth (○—○).

The influence of aeration

It was hoped that an interference with metabolism, such as might be brought about by growth under widely different conditions of aeration, would throw light on the balance of hyaluronic acid synthesis and destruction. The production of hyaluronic acid by strain A111, however, was not influenced by shake or static cultivation (Table 2), despite a prolongation of exponential

growth in shake culture. In completely anaerobic culture growth was slow and poor but 4.9 mg. polysaccharide/mg. bacterial N was present after 24 hr, growth compared with 5.2 mg. for a shake culture of strain A 111 at an equivalent stage

Table 2. *The production of hyaluronic acid by strain A 111 in shake and static culture*

Age of culture (hr.)	Mg. hyaluronic acid/100 ml. of culture			
	Static		Shake	
	1	2	1	2
0	0	0	0	0
1	2.5	2.5	3	2
2	7.3	7.3	6.3	5.7
3	18	17	15.5	13.5
4	34	33	30	26
5	44	44	41	38
6	54	52	56	54
7	66	61	—	72
8	72	72	80	98
24	88	88	80	98

of growth in terms of bacterial N. Furthermore, the production of hyaluronidase by the non-capsulated strain C7 at all stages of growth was not greatly influenced by extreme aerobiosis or anaerobiosis as judged by rough titrations of enzyme by the M.C.P. method (Table 3). The higher titres in young anaerobic culture are probably due to the shorter growth lag. The degree of aeration,

Table 3. *The production of hyaluronidase by strain C7 in aerobic and anaerobic culture*

Age of culture (hr.)	M.C.P. units/ml. of culture			
	Anaerobic		Aerobic	
	1	2	1	2
2	< 12	< 12	< 12	< 12
3	50	25	25	12
4	200	200	100	100
5	400	200	200	400
6	400	400	200	400
7	400	200	400	400
24	1600	400	800	800

however, markedly influenced the balance of hyaluronic acid synthesis and destruction by strain LM (Table 4). As with strain C7 there was a shorter growth lag in anaerobic than in aerobic culture, but this in itself could not account for the faster rate of hyaluronic acid disappearance anaerobically since the difference in the rates of this disappearance was much greater than the differences in the growth lag. Anaerobiosis might select mutants with enhanced hyaluronidase production, as reported by Sallmann (1951) for group A and group C strains whose capsulation was not examined, or reduce the lag in hyaluronidase synthesis. Rogers (1954) has shown that

such a lag, differing from the ordinary growth lag, exists for constitutive hyaluronidase synthesis by some staphylococci. Faber & Rosendal (1954) report a more rapid production of hyaluronidase by group A strains under anaerobic conditions but do not state whether growth rate is also increased. It is also possible that hyaluronic acid synthesis itself in strain LM, unlike that in A 111, is influenced by aeration and that enzyme production is unaffected.

Table 4. *The production of hyaluronic acid and capsules by strain LM in aerobic and anaerobic culture*

Age of culture (hr.)	Capsulation		Mg. hyaluronic acid/100 ml. culture	
	Anaerobic	Aerobic	Anaerobic	Aerobic
2	+	+	3	3
3	+	+	8	13
3½	+	+	10	21
4	+	+	13	28
4½	+	+	5	28
5	+	+	0	26
6	0	+	0	22
7	0	+	0	2
24	0	0	0	0

Measurements of hyaluronidase activity in aerobic and anaerobic cultures of strain LM using the turbidimetric method showed that enzyme activity reaches a maximum towards the end of the log phase of growth, whether aerobic or anaerobic, and then falls quite sharply. Cultures at 24 hr. were much less active than at 6–8 hr. Pike (1948*a*) reported an inexplicable fall in hyaluronidase activity of ageing cultures of some non-capsulated group A and group C streptococci which may have been similar in nature to that described here. In Pike's work, as in this, the fall occurred at neutral pH. The loss of activity is due to the extreme thermolability of strain LM enzyme (MacLennan, 1956).

The spreading activity of strain LM cultures

The spreading factor activity in young strain LM culture supernatants, attributed by Pradhan (1937) to capsular material, might be caused by small amounts of hyaluronidase present. Streptococcal hyaluronidase is a potent spreading factor (McClean, 1941) and, moreover, isolated group C capsular hyaluronic acid has no spreading activity (Seastone, 1939). Furthermore, the fall of spreading activity in old cultures noted by Pradhan and attributed by him to capsule loss is equally explicable by the inactivation of hyaluronidase reported above. To exclude capsular material as the cause of spreading activity it is sufficient to demonstrate this activity in a strain LM culture free from capsules and hyaluronic acid, since the enzyme has already been demonstrated (Table 1) in the younger, capsulated cultures shown by Pradhan to possess spreading activity.

Strain LM was grown in PSB in which serum was replaced by distilled water; the absence of serum increases hyaluronidase activity (Pike, 1948*a*;

MacLennan, 1956). At 8 hr. enzyme activity was maximal, falling to a low value at 24 hr. Hyaluronic acid could not be detected turbidimetrically at 8 hr. and capsules were absent. The 8 hr. culture was centrifuged and the supernatant filtered through a bacterial, sintered glass filter. A portion of the filtrate was heated at 56° for 1 hr. to serve as a control. After the addition of trypan blue (vital) to a concentration of 1 %, 0.25 ml. vol. of the heated and unheated filtrates were injected intradermally into white rabbits. Samples of heated and unheated medium were injected as additional controls. Each preparation was injected into four rabbits at two sites and heated and unheated, but otherwise homologous, preparations were injected into comparable sites on the opposite flanks of each rabbit. It was noted that only the blebs produced by the unheated culture supernatant flattened out quickly. After 1 hr. the rabbits were flayed and the dyed area measured by tracing on to graph paper. Table 5 shows that the 8 hr. culture had a slight but definite, thermolabile, spreading activity. Since the culture was not capsulated and hyaluronic acid was absent this activity can be attributed to the small amount of hyaluronidase present.

Table 5. *The spreading factor activity of a strain LM 8 hr. culture supernatant in rabbit dermis*

Area of dye spread (sq.cm.).						
Rabbit no.	Culture medium	Heated culture medium	Difference	8 hr. culture supernatant	Heated culture supernatant	Difference
1	10.8	9.0	1.8	17.4	15.0	2.4
	9.0	9.2	-0.2	14.4	9.6	4.8
2	10.2	9.6	0.6	14.8	9.0	5.8
	12.2	10.4	1.8	18.6	10.2	8.4
3	13.2	12.4	0.8	19.4	15.8	3.6
	11.8	11.6	0.2	20.8	12.2	8.6
4	14.8	10.0	4.8	14.8	11.8	3.0
	11.0	12.6	-1.6	16.2	10.2	6.0

Mode of hyaluronidase production by strain LM

The production of hyaluronidase by strain LM might be due to a substrate induced (adaptive) enzyme synthesis or to the selection of hyaluronidase producing mutants. That variants occur is evident from the observation that strain LM, as revived from an NCTC stock culture on to moist, 20 % horse serum, 1 % glucose agar plates, yields two distinct colony types, mucoid and non-mucoid, containing respectively capsulated and non-capsulated cells. The two variants bred true on successive subculture. Grown in PSB with continual neutralization, capsulated organisms were produced only by the mucoid variant. Hyaluronidase was, however, produced by both variants from early growth onwards. Enzyme titres were similar, although the enzyme was formed rather later by the non-capsulated variant. On plating 24 hr. cultures mucoid colonies alone were obtained from the mucoid cultures and only non-mucoid colonies from the non-mucoid cultures. Fifty mucoid colonies were grown

separately in PSB; hyaluronic acid was absent from all cultures after 24 hr. growth.

It appears that capsulated cells are able to form hyaluronidase and that, although non-capsulated cells also produce the enzyme, the destruction of hyaluronic acid in cultures of the mucoid form is not due to the selection of non-capsulated variants. There was no evidence that some capsulated cells were unable to produce hyaluronidase. In view of the similar degree of enzyme production by capsulated and non-capsulated organisms it appears that the non-capsulated state is due to a loss of ability to synthesize capsular material rather than to the suppression of the capsule by enhanced enzyme activity. It also appears that enzyme formation is not stimulated by the presence of its substrate since were this so the capsulated organism would be expected to produce hyaluronidase in relatively large amounts in response to the presence of a high local concentration of hyaluronic acid in the capsule.

I wish to thank Dr D. McClean for his guidance and encouragement in this work which formed part of a Ph.D. thesis submitted to the University of London, 1954.

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The Isolation and Characterization of a Hyaluronidase Produced by a Capsulated Strain of Group C Streptococcus

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SUMMARY: A hyaluronidase preparation was isolated by the methods of protein precipitation from the culture fluid of a capsulated group C streptococcus cultivated in a serum-free medium. The preparation was active in the mucin clot prevention and turbidimetric tests for hyaluronidase and rapidly destroyed the capsules of group A and group C streptococci. The enzyme resembled other streptococcal hyaluronidases in its reaction to pH changes but differed from these both in its thermolability and antigenic specificity.

Hyaluronidase activity in cultures of capsulated or hyaluronic acid producing streptococci has previously been detected only by prolonged incubation of these cultures, or their filtrates or supernatants, with hyaluronic acid (Pike, 1948*b*; Quinn, Seastone & Weber, 1953; Faber & Rosendal, 1954). Little is known of the properties of the agent responsible for this activity beyond the fact that it is thermolabile and filterable (Pike, 1948*b*). However, the sera of patients with group A streptococcal infections often contain antibody to group A hyaluronidase, although the infecting organism produces hyaluronic acid and little or no hyaluronidase *in vitro* (Quinn, Seastone & Weber, 1953). This is further evidence that traces of hyaluronidase are formed in ageing cultures of capsulated group A streptococci and are responsible for the destruction of hyaluronic acid. The relatively high hyaluronidase activity of a capsulated group C strain reported by MacLennan (1956) suggested that this enzyme might be concentrated by protein precipitation and its properties more fully examined.

MATERIALS AND METHODS

Source of streptococci. National Collection of Type Cultures (NCTC). Strain LM (NCTC 6176) is a capsulated, hyaluronic acid and hyaluronidase producing group C strain. Strain C7 (NCTC 4540) produces hyaluronidase to high titre (Rogers, 1944) and is not capsulated at any stage of growth (MacLennan, 1956).

Cultivation of streptococci. Cultures were made as described by MacLennan (1956). They were grown from heavy inocula, saline washed, in broth at 37°, with continual neutralization by sodium hydroxide. After unsuccessful attempts to obtain preparations with high hyaluronidase activity by ammonium sulphate precipitation of supernatants from cultures in the serum enriched medium PSB (MacLennan, 1956) serum was replaced by distilled water. The serum-free medium PB is thus identical with PSB in the content

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and concentration of nutrients other than those present in serum. Despite a 25 % decrease in growth in PB cultures hyaluronidase activity by the turbidimetric test (MacLennan, 1956) was greater than that of PSB cultures at all stages of growth. The non-specific hyaluronidase inhibitor present in serum (Hobby, Dawson, Meyer & Chaffee, 1941; McClean, 1942) probably lowers the activity of PSB cultures; it is unlikely, in view of Pike's (1948*a*) work with non-capsulated streptococci, that the actual production of hyaluronidase is influenced by serum.

Preparation of strain C7 hyaluronidase. The formation of this enzyme is enhanced by cultivating the non-capsulated strain C7 in the presence of hyaluronic acid (Rogers, 1944). Cultures were grown, therefore, for 24 hr. in 0.1 % hyaluronate enriched PB (Rogers, 1946) for 24 hr. with continual neutralization by 10 % sodium hydroxide. The cells were discarded after centrifugation and thiomersalate added to the supernatant to a concentration of 1/10,000. This preparation is 'crude C7 hyaluronidase'. Ammonium sulphate and ferric hydroxide purified C7 hyaluronidase were prepared from culture supernatants by the methods described by Rogers (1946). Both contained 1/10,000 thiomersalate.

Preparation of testicular hyaluronidase. Adult rabbit testes, after removal of fat, were finely minced with an equal volume of distilled water and the suspension centrifuged. The supernatant was diluted to an activity of 500 M.C.P. units/ml., dispensed in 1 ml. amounts into ampoules and dried from the frozen state.

Measurement of hyaluronidase and hyaluronidase antibody. In addition to the sensitive turbidimetric test for the detection of small amounts of hyaluronidase (MacLennan, 1956) the turbidimetric test of Humphrey & Jaques (1953) and the mucin clot prevention (M.C.P.) test of McClean (1943) were used.

Antibody to hyaluronidase was titrated by the method of McClean (1943) using the M.C.P. test and by a turbidimetric method described below for the measurement of non-specific hyaluronidase inhibitors. All sera were heated at 56° for $\frac{1}{2}$ hr. before testing to destroy the non-specific, thermolabile inhibitor of hyaluronidase present in mammalian sera. Enzyme-antibody mixtures were allowed to react overnight at room temperature before measuring residual enzymic activity since neutralization of hyaluronidase by its antibody continues for some hours after mixing as noted first by Faber (1953). The activity of a serum was expressed as the number of M.C.P. units neutralized by a 1/20 dilution of the serum tested, and as the number of turbidity reducing units (T.R.U.) neutralized by 1 ml. of undiluted serum.

Measurement of non-specific hyaluronidase inhibitors. The inhibition of hyaluronidase by sodium azide, sodium fluoride, phosphorylated hesperidin and rehibin was measured turbidimetrically (Humphrey & Jaques, 1953) by Faber's method (1953) for the titration of hyaluronidase antibody. Inhibitor potency was recorded as the concentration in mg./ml. inhibiting 1 T.R.U. hyaluronidase.

Two series of enzyme dilutions in gelatin buffer were prepared to contain 0.5, 1, 1.5, 2, 3, and 4 T.R.U./ml. 0.2 ml. of aqueous dilution of inhibitor was

added to 0.8 ml. vol. of one series of dilutions and 0.2 ml. of water to the other. After $\frac{1}{2}$ hr. at room temperature 0.8 ml. of 0.08 % hyaluronic acid (Allen and Hanburys Ltd.) was added and the mixtures incubated for 30 min. at 37°. A 30 min. incubation was preferred to the 10 min. recommended by Humphrey & Jaques (1953) because of the weak activity of the strain LM enzyme. After incubation the tubes were chilled and 0.2 ml. of inhibitor solution added to the controls and 0.2 ml. of water to the experimental series immediately before precipitating residual hyaluronic acid with acid serum. This second addition of inhibitor was necessary because of the high colour of some inhibitor solutions which would otherwise cause differences in the colorimeter readings of control and experimental tubes.

Hyaluronidase antibody was also titrated by this method as a check on the M.C.P. test.

RESULTS

Thirty litres of a strain LM culture in PB were treated with thiomersalate, 1/10,000, after cultivation with continual neutralization for 7½ hr., the period found by trial for maximum hyaluronidase activity. The supernatant was siphoned off and 15 kg. ammonium sulphate added. The scum that formed was skimmed off, centrifuged and dialysed against running tap water until free from ammonium sulphate. A 150-fold decrease in volume was obtained. This preparation had an M.C.P. titre (20 min. incubation) of 40 units and a turbidimetric titre (30 min. incubation) of 19 T.R.U. This is the first demonstration of hyaluronidase activity in material from a culture of a capsulated streptococcus by conventional, quantitative methods of hyaluronidase assay. In the turbidimetric test the shape of the curve relating enzyme concentration to substrate destruction was very similar to that given by strain C7 hyaluronidase. The preparation, in common with strain C7 and testicular hyaluronidases, very rapidly decapsulated young cultures of strain LM and a group A strain. Enzymic activity was completely destroyed by 24 hr. at 37° and fell 50 % after 14 days at 22°. The preparation was therefore dried from the frozen state and stored at 2°. After unsuccessful attempts to purify the enzyme by adsorption on ferric hydroxide (Rogers, 1946) and calcium phosphate at pH 7.0 it was decided to characterize the enzyme in the impure concentrate in the hope that information thus acquired might suggest means of increasing the amount of hyaluronidase produced in culture. Purification could then be attempted on more active crude material.

The properties examined were the pH optimum and inactivation, the influence of temperature on stability, antigenicity and inhibition by fluoride, azide, phosphorylated hesperidin and rehibin. These properties were compared with those of the hyaluronidase produced in large quantities by the non-capsulated group C streptococcus, strain C7.

pH optimum and pH inactivation

It was not possible to use standard methods of hyaluronidase assay for enzyme-substrate mixtures over a wide range of pH values. McIlvaine buffers, M/30 with respect to the phosphate component, were found to maintain the

original pH of enzyme + substrate mixtures during enzymic destruction of the substrate at 37°. The sodium chloride content of the buffers was M/3 which gave optimal turbidity on precipitation of hyaluronic acid with acid serum at 0°.

The reaction mixture comprised 0.2 ml. aqueous enzyme dilution, 1.8 ml. M/30 McIlvaine buffer, M/3 with respect to NaCl and 2.0 ml. aqueous hyaluronic acid solution, 0.4 mg./ml. The enzyme was added last. Reaction mixtures with buffers of various pH values were prepared in triplicate, one series being used to confirm that the measured pH of the mixtures did not change during incubation at 37° for 30 min. The residual hyaluronic acid after incubation was precipitated at 0° with 4 ml. acid serum in the remaining two series of reaction mixtures. Turbidities were developed for $\frac{1}{2}$ hr. at room temperature before reading on the Evans' Electroselenium Co. Ltd. (EEL) colorimeter, filter 622. Calibration curves were prepared from dilutions of the standard hyaluronic acid solution at each pH and from these curves the amount of hyaluronic acid destroyed was read off. At pH 7.0 turbidity was proportional to polysaccharide concentration and the amount of hyaluronic acid destroyed was proportional to enzyme concentration. The enzyme concentrations used were such that a slight change in concentration produced a large change in substrate destruction.

The pH optimum of strain LM hyaluronidase was compared with that of a strain C7 enzyme, dilutions having similar activity at pH 7.0 being used. Both were optimally active at pH 6 with a rapid fall below 5.6 and above 6.6 (Fig. 1). This observation agrees with those of Hale (1944) and Rogers (1948) for streptococcal hyaluronidases.

To measure pH inactivation enzyme preparations were adjusted to a series of low and high pH values by the addition of strong acid and alkali respectively. After $\frac{1}{2}$ hr. at room temperature samples were neutralized with dilute acid or alkali and diluted to a constant volume. Residual enzymic activity was measured on 0.2 ml. samples by the method described for pH optimum measurements, using buffer at pH 7.0. Both enzymes lost activity greatly below pH 5 and above pH 8.

The influence of temperature on streptococcal hyaluronidases

There is no doubt that the streptococcal and pneumococcal hyaluronidases are more thermolabile than the testicular enzyme (Harris & Harris, 1950; Meyer & Rapport, 1952).

Thermolability was determined by heating 0.5 ml. vol. of aqueous enzyme dilutions at various temperatures for 15 min. and measuring residual activity on 0.2 ml. samples incubated with buffered substrate at pH 7.0, for 30 min. at 37°. As an additional precaution 0.4% gelatin was included in the buffer since Rogers (1948) reported that gelatin, peptone and gum arabic protect hyaluronidases at ordinary temperatures. It was hoped by this means that enzyme preparations differing in protein content would be equally protected in the test.

The strain C7 enzyme, whether 'crude C7 hyaluronidase' or a protein concentrate prepared as described for strain LM, was destroyed between the

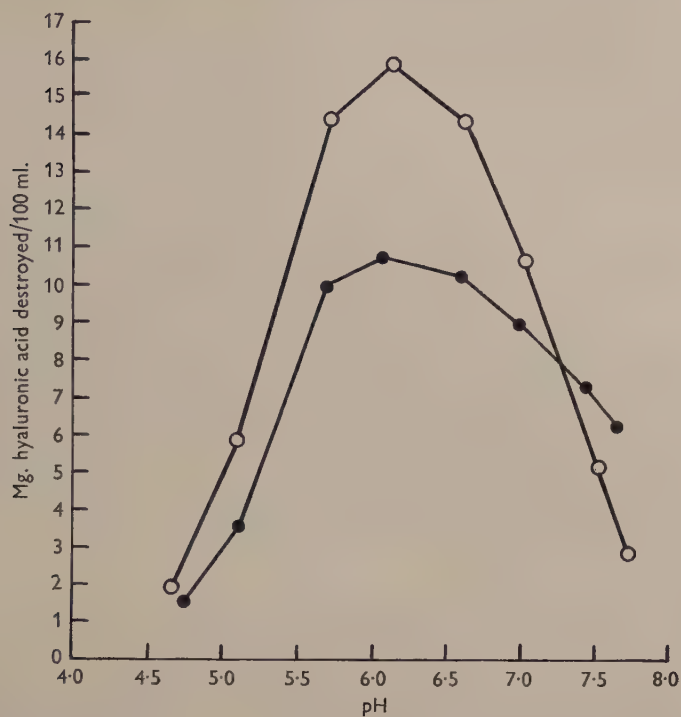


Fig. 1. The pH optima of strain C7 (○—○) and strain LM (●—●) hyaluronidases.

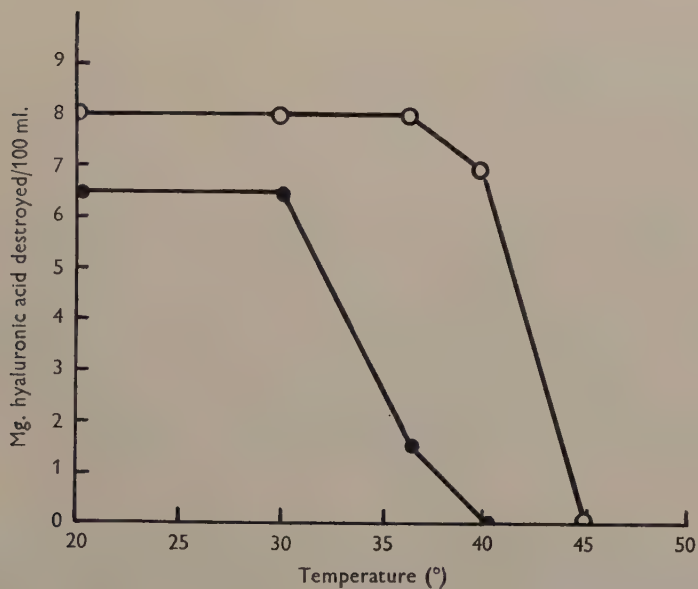


Fig. 2. The thermal destruction of strain C7 (○—○) and strain LM (●—●) hyaluronidases.

temperature limits 36·5° and 45° (Fig. 2). This result agrees with the observations of Harris & Harris (1950) on the rate of inactivation of pneumococcal and group A streptococcal hyaluronidases at 42°. Strain LM enzyme, on the other hand, was destroyed between the temperature limits 30° and 40°. This was confirmed several times; there is little doubt that this enzyme is more thermolabile than any previously described hyaluronidase.

The fall in hyaluronidase activity of strain LM cultures after growth has ceased (MacLennan, 1956) and in culture supernatants incubated at 37° in the presence of thiomersalate appeared now to be explained by the thermolability of the enzyme. The possibility remained, however, that the strain LM cultures, unlike those of strain C7, contained an agent that destroys hyaluronidase, for example a protease of wide substrate specificity such as is found in the culture supernatants of some group A streptococci (Elliott, 1945; Mycek, Elliott & Fruton, 1952). If this hypothetical agent resembled the protease described by Elliott in being more active at 37° than at 22°, then the rapid destruction of strain LM enzyme at 37° might be explained. To test this possibility the thermolability of strain LM hyaluronidase alone and in combination with strain C7 or testicular hyaluronidase was determined.

Table 1. *The thermolability of strain LM enzyme, alone, and in the presence of other hyaluronidases*

Hyaluronidase preparation	Mg. hyaluronic acid destroyed/100 ml.			
	Unheated		Heated 40° for 15 min.	
	1	2	1	2
1 vol. water + 1 vol. strain LM hyaluronidase	2·5	2·5	0	0
1 vol. water + 1 vol. strain C7 hyaluronidase (crude)	3	3	3	2·5
1 vol. water + 1 vol. testicular hyaluronidase	3·5	4	3·5	4
1 vol. C7 + 1 vol. LM	7	7	3	2·5
1 vol. testicular + 1 vol. LM	8	8	3·5	3

Strain LM enzyme and the two mixtures were heated at 40° for 15 min. The activity before and after heating was compared with that of the separate enzyme at concentrations equal to their individual concentrations in the mixtures. Table 1 shows that the enzyme activity of the heated mixtures could be attributed to that of the testicular or strain C7 enzyme components alone. A protease would presumably have destroyed all three enzymes indiscriminately. Since this did not occur the selective destruction of strain LM hyaluronidase is most probably due to its high thermolability.

The antigenicity of streptococcal hyaluronidases

Hobby *et al.* (1941) found that antiserum to pneumococcal hyaluronidase inhibited this enzyme but was inactive against streptococcal hyaluronidases. McClean (1943) reported that antiserum to strain C7 streptococcal hyaluronidase

neutralized other group C enzymes of types, 7, 20 and 21 but was inactive against group A, type 4. He suggested that the streptococcal enzymes were group specific but not type specific. Wenner, Gibson & Jaques (1951) have found that the hyaluronidases of streptococcal groups A, B and C are serologically distinct, whereas those of groups C and G are not.

Sterile filtrates (sintered glass filters) of strain LM and strain C7 enzymes were used to prepare antisera. The C7 enzyme preparations were purified by ammonium sulphate precipitation or by adsorption on $\text{Fe}(\text{OH})_3$. A crude preparation was also used. 0.5 ml. vol. were injected subcutaneously at weekly intervals into pairs of rabbits for 4 weeks and 1.0 ml. vol. by the same route after 3 weeks rest for a further 3 weeks.

Table 2. *The immunization of rabbits with streptococcal hyaluronidase preparations*

Antigen		No. of M.C.P. units neutralized by 1/20 antiserum								
Preparation	Unitage M.C.P./ml.	Rabbit no.	Bleeding							
			Control	3rd	4th	5th	6th	7th	8th	9th
C7 enzyme (crude)	750	1	0	0	—	16	4	32	32	32
		2	0	1	—	32	16	64	64	64
C7 enzyme (ammonium sulphate)	1800	3	0	2	8	8	16	128	128	—
		4	0	1	32	64	16	64	64	—
C7 enzyme (ferric hydroxide)	500	5	0	0	—	2	2	8	32	16
		6	0	0	—	1	1	4	4	16
LM enzyme	22	7	0	0	—	0	0	6	6	3
		8	0	0	—	3	6	6	6	4

Table 2 reveals that all 3 strain C7 preparations produced a good antibody response as measured by the M.C.P. method. The $\text{Fe}(\text{OH})_3$ purified enzyme was the least effective, probably due to its low M.C.P. activity. Antisera to strain LM enzyme neutralized this enzyme but strain C7 antiserum did not neutralize strain LM enzyme or vice versa. This is the first report that hyaluronidases with different antigenic specificities are produced by streptococcal strains of the same group. Testicular and group A, type 4 streptococcal hyaluronidase were not neutralized by strain LM antisera either (Table 3). Since antisera to

Table 3. *The specificity of streptococcal hyaluronidase antisera*

Enzyme preparation tested	No. of M.C.P. units neutralized by 1/20 serum			
	Antiserum to C7 enzyme (ammonium sulphate)		Antiserum to LM enzyme	
	Rabbit 3	Rabbit 4	Rabbit 7	Rabbit 8
C7 Enzyme (ammonium sulphate)	80	40	0	1
LM enzyme	0	1	3	4
Group A, type 4 enzyme (crude)	0	1	0	0
Testicular enzyme	0	1	1	0

hyaluronidase produced *in vivo* by capsulated group A strains neutralize group A, type 4 enzyme it is clear that there is not a characteristic single hyaluronidase produced by capsulated streptococci irrespective of serological group.

Confirmation of the cross-neutralization tests was obtained from the turbidimetric method (Table 4). There was a slight neutralization of strain LM enzyme by the strain C7 antiserum.

Table 4. *The specificity of streptococcal hyaluronidase antisera in a turbidimetric test*

Enzyme preparation tested	No. of T.R.U. neutralized by 1 ml. undiluted serum			
	Antiserum to C7 enzyme (ammonium sulphate)		Antiserum to LM enzyme	
	Rabbit 3	Rabbit 4	Rabbit 7	Rabbit 8
C7 enzyme (ammonium sulphate)	600	610	0	0
LM enzyme	20	40	50	240

Hyaluronidase inhibitors

Weiner & Seastone (1951) observed that the normal process of capsule loss by group C streptococci in suspension was inhibited by azide and fluoride ions, although these did not prevent capsule destruction by streptococcal hyaluronidase. This implies that capsule loss may be caused by an enzyme other than hyaluronidase, but Weiner & Seastone were unable to isolate such an enzyme.

Since strain LM hyaluronidase is present in young capsulated cultures of this organism (MacLennan, 1956) and a protein concentrate of the enzyme can rapidly decapsulate streptococci there is little doubt that it plays a part in normal capsule loss. The action of fluoride and azide on this enzyme, and on strains C7 and testicular hyaluronidases, was examined by the turbidimetric test (Humphrey & Jaques, 1953) to measure inhibition in the manner suggested by Faber (1953).

Concentrations of 0.02 M-fluoride and azide in enzyme + inhibitor mixtures had no influence on enzyme action. It is possible that the decapsulating action of strain LM enzyme is inhibited by fluoride and azide; this was not investigated.

The action on strain LM and other hyaluronidases of rehibin, a polymerized derivative of gentisic acid (Hahn, 1952), and of phosphorylated hesperidin (Beiler & Martin, 1948), two inhibitors of testicular hyaluronidase, was examined. Both substances were powerful inhibitors of the testicular enzyme in the turbidimetric test and rehibin at fairly high concentration inhibited the strain C7 enzyme also (Table 5). Neither substance had any action on strain LM hyaluronidase but this may be due to the high protein concentration of this preparation since inhibition of the testicular enzyme diluted twofold in heated, inactive strain LM enzyme was considerably reduced. The inhibition of hyaluronidase by flavonoids such as hesperidin is not specific, other enzymes being inhibited (Rodney *et al.* 1950) and therefore the presence of other proteins

in hyaluronidase preparations might well reduce inhibition by combining with the inhibitor. The results with strain LM hyaluronidase are therefore inconclusive.

Table 5. *The inhibition of hyaluronidases in the turbidimetric test*

Enzyme preparation tested	Concn. of inhibitor in mg./ml. required to inhibit 1 T.R.U. enzyme	
	Rehibin	Phosphorylated hesperidin
Testicular enzyme	0.033	0.042
	0.036	0.055
Strain C7 enzyme (ammonium sulphate)	0.4	No inhibition
	0.37	at 0.5
Strain LM enzyme	No inhibition	No inhibition
	at 0.5	at 0.5

I wish to thank Dr D. McClean, Dr H. J. Rogers and Dr D. E. Dolby for guidance and encouragement in this work, Dr Rogers for a gift of rehibin and Dr Beiler of the National Drug Co., Philadelphia, U.S.A., for a gift of phosphorylated hesperidin. This work formed part of a Ph.D. thesis submitted to the University of London, 1954.

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A Rapid Method for Determining the Proportion of Viable Bacteria in a Culture

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SUMMARY: A method of making graticules on the surface of Cellophane is described. The proportion of viable bacteria in a culture can be estimated by inoculating a sample on to such a graticule, and counting the organisms before and after a short period of growth.

The usual plating-out method of obtaining a viable count of bacteria is expensive of both time and materials, and the result is obtained only after a period of incubation never less than 12 hr. The result could obviously be established more quickly by counting the organisms in an inoculum under the microscope, and again counting those which had multiplied after a relatively short period of growth. The practical difficulty in doing so is that only a few organisms can comfortably be accommodated in a single field of the microscope, if confusion of one micro-colony with another is to be avoided. Some form of counting-chamber is required, to enable a definite, recognizable, and sufficiently large area to be scanned before and after the growth period. It is now well known that most common bacteria will grow freely on the surface of a Cellophane membrane through which they draw their nourishment, and Pearce & Powell (1951) and Harris & Powell (1951) have shown also that Cellophane is particularly suitable as a support for growing organisms under the microscope. A rapid method for estimating a viable count, described in the following paragraphs, consists in growing the organisms on a Cellophane membrane lying on the surface of a suitable agar medium. Its practicability depends on a simple device for engraving a graticule on the membrane, so as to meet the difficulty mentioned above. The development of this method was occasioned during the study of continuous culture of the Monod (1950) type. Most of the variables of interest in such a study (e.g. pH, temperature, flow rate) can be determined almost instantaneously, and applied to automatic monitoring and regulation of the culture. This cannot yet be done with the viable count, but the early detection of changes in viability is still a great economy in the correction of unforeseen faults and in determining the effects of experimental variation in working conditions.

Construction and use of the engraving tool

Razor blades of about 100 μ . thickness are marketed by several manufacturers (Thin Gillette blades were used here). A pile of such blades, impressed twice on a Cellophane surface, one impression at right angles to the other, produces a

grid of which one square is conveniently covered by a single field of a 4 mm. microscope objective with a $\times 10$ eyepiece. The blade edges are not straight, but have a gentle undulation with an amplitude of $10\text{--}20\mu$. in the plane of the blade; a tolerably uniform grid can only be produced if the blades are mounted so that the edges are in register, within a few microns, over the length of the graticule edge (1 mm. is sufficient). The required accuracy of setting is achieved

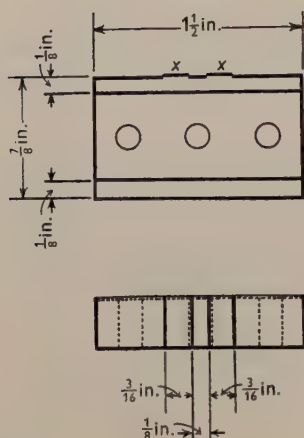


Fig. 1



Fig. 2

Fig. 1. Mild steel block. Two are used for clamping the pile of razor blades. The lands *xx*, *yy* project about 0.005 in. above the neighbouring surface.

Fig. 2. Preparation for mounting blades. *C*, body of magnetic chuck; *R*, rollers. Viewed from above.

by clamping the blades between two mild steel blocks of the form shown in Fig. 1. These blocks are of approximately the same size (in side elevation) as the blades; on two surfaces of each, lands *xx*, *yy* are left about 0.005 in. proud of the neighbouring surface. After being machined, the two blocks are bolted together and the lands *xx* finish-ground so that all four are in the same plane.

The blades are positioned as follows: two silver steel rods $1\frac{1}{2}$ in. long and $\frac{1}{8}$ in. diameter, ground and polished, are clamped to the surface of a magnetic chuck with their axes vertical, approximately parallel and $\frac{3}{16}$ in. apart. They are prevented from rolling by an application of plasticine to their ends. One of the blocks is then placed with its lands *xx* in contact with the lower ends of the rollers and the lands *yy* upwards (Fig. 2); the field of the chuck is sufficient to hold it in position. The blades, conveniently eleven in number, are placed one by one on the block; again the field keeps their edges in contact with the rollers. Some delicacy of touch is needed to prevent the edges being turned by longitudinal rubbing. The second block is then similarly brought into contact with the upper ends of the rollers, and slid downwards upon the stack

of blades. The whole pile is bolted firmly together, and the blade edges between the lands are then in the same plane as the lands themselves.

The second member of the tool, the anvil, which determines the depth of the impression, consists of a glass plate painted with label varnish which is scraped away except for a transverse strip $\frac{3}{32}$ in. wide. The correct thickness, 10–12 μ , must be reached by trial, the varnish being diluted when necessary. It can be estimated quite accurately enough on an ordinary micrometer. The plate is hinged by means of adhesive tape to one edge of the working face of the pile so that the strip of varnish lies opposite the gap between the lands *xx* (Pl. 1*a*).

The pile must be washed in light petroleum before use, in order to remove grease and dust.

A graticule is made by placing a disk of Cellophane centrally on the pile of blades, bringing down the glass plate over it, and pressing fairly heavily with the thumb; this is repeated after turning the disk through a right angle. The disk is marked at some point on its edge so that it can be brought into the same azimuth whenever it is examined under the microscope. Under a low power (Pl. 1*c*) the graticule looks unpromisingly dirty; this is due to the small aperture of the objective, which increases contrast and, because of the large focal depth, shows up every inequality within the thickness of the membrane and part of the underlying agar. At a higher power (Pl. 1*d*), the surface is seen to be mostly free from gross irregularity. Such a graticule can be made in about 1 min., inclusive of punching out the disk, and so it can economically be discarded after use. The useful life of a pile of blades is not known—many hundred impressions at least can be made without loss of edge.

Ordinary commercial Cellophane is suitable for this application (no. PT. 300, Messrs British Cellophane Ltd., Bridgwater, Somerset). As it comes from the manufacturer it is physically clean and sterile over large areas; for the present purpose freedom from grease and dust is much more important than asepsis, so the Cellophane should be kept wrapped and handled only with forceps. When slow-growing organisms are to be examined, contamination can in nearly every instance be avoided by washing the completed graticule in boiling water and laying it while still wet on the agar plate.

Method for determining the proportion of viable organisms in an inoculum

To make a count, a graticule with the engraved side uppermost is placed on the surface of a nutrient agar plate. The centre of the disk is inoculated by means of a 1 mm. loop with a suspension of organisms diluted to 10^6 – 10^7 /ml. The inoculated area is examined under vertical illumination with a dry 4 mm. metallurgical objective. To hasten drying and permit the organisms to be seen clearly, a stream of air is blown over the surface; Pl. 1*b* shows a jet clipped to the objective mount for this purpose. When all the liquid is evaporated, the current of air is decreased as far as possible without allowing the liquid diffusing through the Cellophane to flood the individual organisms (Pl. 1*d*; cf. Harris & Powell, 1951). The graticule is scanned square by square; the number

of single organisms and of groups in each is noted down. Squares in which the count is embarrassed by dirt, objects of doubtful character, overcrowding, or blemishes in the 'Cellophane', can be omitted. After incubation for 2-6 hr. (depending on the organism) the numbers of groups of organisms in each square are again counted. The difference in the group count is the number of viable organisms among the single individuals originally present. This method of counting is adopted because non-viable cells sometimes undergo lysis and leave little residue. If single organisms only were counted, such cells would be recorded as viable. The proportion of viable organisms so obtained must of course be supplemented by a conventional total count if a measure of their actual concentration is required.

Much the best results are obtained if these operations are performed throughout at the temperature of incubation. The dilution and inoculation of the sample at that temperature keeps the lag period to a minimum and the more rapid drying of the inoculum reduces the risk of damage to the organisms. There is a danger that liquid may diffuse to the surface of the 'Cellophane' in sufficient quantity to permit the organisms to move during incubation. This can be avoided by drying the agar plates for 2 hr. at 37° beforehand, and incubating with the lids slightly ajar.

In practice it is found to be nearly always quite easy to distinguish organisms from foreign bodies, though some previous experience is of course desirable. It is impossible to convey in a photomicrograph the nuances obtainable by minute adjustments of focus and of degree of dryness. It is rare to find pairs of organisms in close juxtaposition but not touching. As the inoculum dries, organisms which are only a few diameters apart are drawn together by surface tension. Unless the inoculum is very dense (e.g. > 10 organisms/square), the error which can arise by fusion of adjacent colonies is thus small.

Appraisal of the method

The method has been applied so far to a limited range of organisms only: *Escherichia coli*, *Pasteurella pestis*, *Serratia marcescens*, *Pseudomonas aeruginosa*, several *Bacillus* species; but organisms of many other genera are known to grow satisfactorily on Cellophane. The numerical results are reproducible and agree (except in the special cases discussed below) with those obtained by the normal procedure when due allowance for sampling fluctuations is made (Table 1). There are four principal advantages, apart from speed: (i) Directness—the organisms capable of growing in the circumstances of the test are actually seen to do so; viability is not asserted as an induction from some concomitant property. (ii) A test sample can be transferred within a minute to the graticule. At most one dilution (which can usually be guessed) is needed to bring the organisms to a density convenient for counting. If a culture containing many ill-nourished or unhealthy organisms is subjected to a lengthy process of dilution, some will die meanwhile, whereas rapid transfer to fresh medium leaves less opportunity for irreparable damage to occur. (iii) Errors due to clumping of the organisms are avoided. (iv) Those organisms (especially

certain *Bacillus* spp.) which form diffuse or spreading colonies and cannot be satisfactorily counted by the ordinary method, offer no special difficulty (Table 1).

Table 1. *Proportion of viable organisms in various cultures, estimated by counting on 'Cellophane' gratricules*

Nature of inoculum	Period of incubation (hr.)	No. of single organisms counted	No. of viable organisms	% viable	% viable by plating-out (50)
Artificial mixture of 50% viable, 50% heat-killed <i>Escherichia coli</i>	2	729	376	52	
<i>Bacillus anthracis</i> spores.	2½	86	79	92	—
		245	229	94	
		122	109	89	
		65	65	100	
		102	96	94	
		76	65	86	
Total of previous six counts	—	696	643	92	90
<i>Pasteurella pestis</i> .	5	154	131	85	—
		128	101	79	
		124	114	92	
		141	127	90	
		137	112	82	
		150	133	88	
Total of previous six counts	—	834	718	86	90
<i>Bacillus cereus</i> spores.	3	213	209	98	c. 50
A strain giving spreading colonies.		239	222	93	(impossible to count accurately)
		219	214	98	
		108	101	94	
<i>Bacillus globigii</i> spores incorporating radio phosphorus.	3	473	30	6.3	7.0
		309	22	7.1	
<i>Aerobacter aerogenes</i> samples from continuous culture.	2	121	106	88	74
		125	109	87	
		151	132	87	

Notes (1) For the purposes of this trial, many of the above suspensions were diluted more than is normally necessary.

(2) *Pasteurella pestis* was grown on a tryptic meat medium with an addition of 0.075 % sodium bisulphite. The individual counts are discordant (in the statistical sense) at the 5 % level of significance. A similar lack of reproducibility occurs in the conventional method of counting, and is no doubt connected with the difficulty of establishing growth from single isolated cells (Herbert, 1949).

(3) A differential staining method (Powell, 1950) showed that about 95 % of the *Bacillus cereus* spores tested were ungerminated, and presumably viable.

(4) For preparation and properties of spores containing ³²P see Harper & Morton (1952).

The main disadvantages are: (i) The limited statistical accuracy. Only a few hundred organisms can conveniently be counted on one graticule. However, it is often possible to inoculate several gratricules on one agar plate at the same time. (ii) Some organisms, ultimately capable of reproduction, may have a very long lag period on transfer to a new medium, and so may be accounted

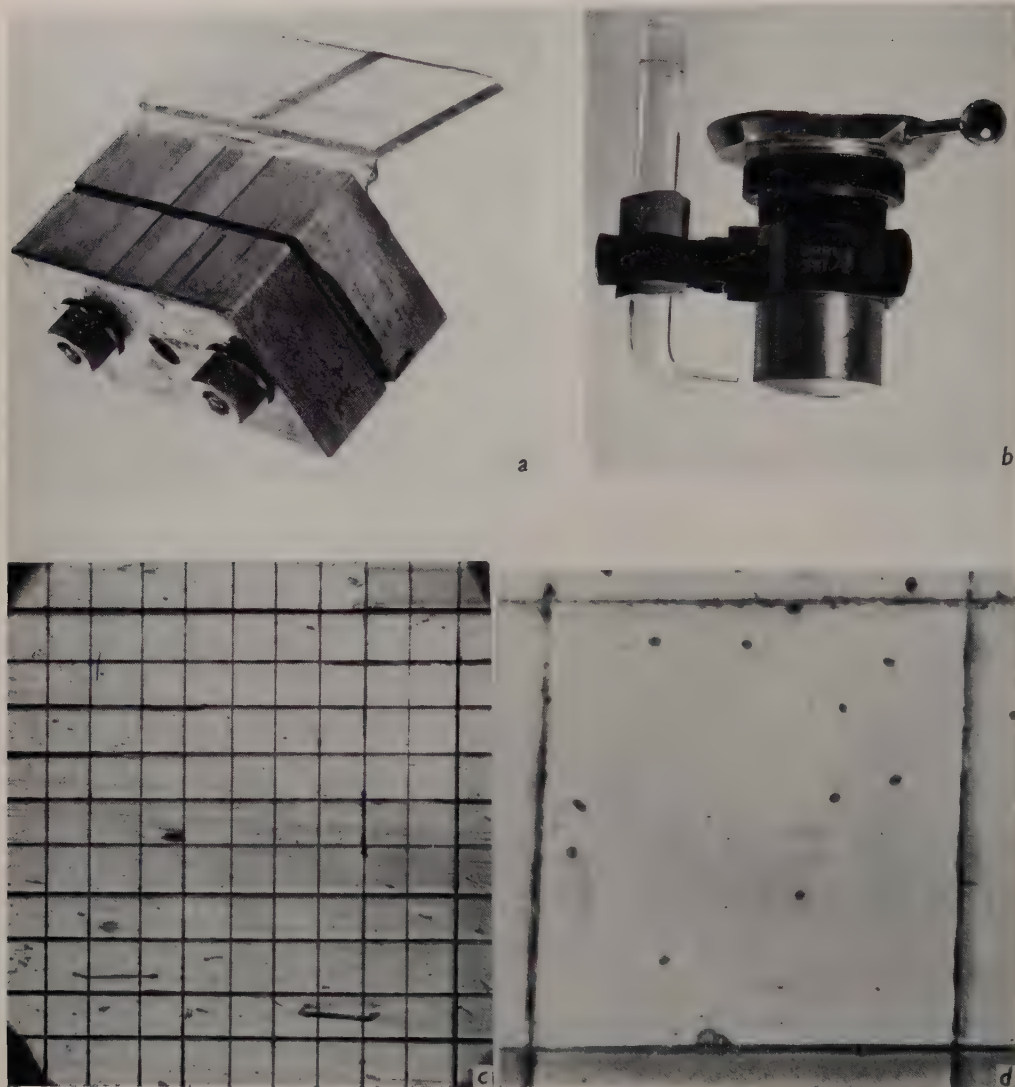
non-viable. Increasing the period of incubation is of no avail if the dispersion of lag is large, because the colonies formed from the organisms first to grow soon extend over a wide area. (iii) Some familiarity with the appearance, under vertical illumination, of different sorts of organisms and of common foreign particles is necessary if the observer is to have confidence in his assessment.

There have been proposed or implied several rapid methods of determining viability based on properties known to be highly correlated with the power of reproduction, e.g. differential staining (Strugger, 1948; Wade & Morgan 1954); refractive index changes, visible under phase-contrast (Barer, Ross & Tzaczky, 1953); growth without division (Valentine & Bradfield, 1953, 1954). Such methods work well with artificial mixtures of unequivocally living and dead organisms, but in natural mixtures there are often many intermediate forms which cannot be categorized with certainty in this way. This is the chief justification for introducing a method which requires specialized apparatus and which, though expeditious, must be painstakingly carried out if it is to yield reliable results.

It seemed at first that counting on the Cellophane surface would be facilitated by applying Valentine & Bradfield's principle, i.e. by incorporating urea in the medium, so that division would be suppressed and viable organisms easily recognizable as long filaments. Trials with several species of organisms gave uniformly low results, evidently because evaporation at the surface raises the urea concentration to a toxic level there (cf. Harris & Powell, 1951).

The Cellophane graticule technique has been found to give higher figures than the usual plating out in three sets of circumstances. (Compare also the discussion of Valentine & Bradfield, 1954.) (i) In samples taken from continuous cultures of the Monod type (Table 1). The discrepancy is probably to be ascribed to the death of some organisms during the preparation of serial dilutions. In such cultures the growth-rate is controlled by limiting the supply of nutrient, i.e. by starving the organisms, so that they can be expected to have but little reserve of energy. The viability as estimated by the differential stain of Wade & Morgan (1954) is even higher—nearly 100%. (ii) Organisms which have been damaged by radiation may reproduce for a few generations, but never give rise to macroscopic colonies (Buckland, Harper & Morton, 1950). (iii) In non-homogeneous cultures two organisms of slightly different growth-rates will give rise to colonies of which one may have reached a nearly static condition before the other has become visible. After only a few generations of growth the difference will not be clearly evident. A preparation of highly virulent spores of *Bacillus anthracis* gave an apparent viability of only 0.1% by the usual method. Closer examination of the plates showed that many microscopic colonies accompanied the few of normal size. In this instance it is likely that the much higher figure obtained by growth on Cellophane was still too low.

Anomalies of this kind arise not from any genuine inconsistency, but because it is practically impossible in the present state of knowledge to set up a satisfactory definition of viability; for this reason no two ways of deter-



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(Facing p. 159)

mining it will agree in every application. In principle, viability only has meaning with respect to an environment which can be specified. It is frequently unnecessary to take account of the restriction, but signal reminders occur from time to time, as in 'dormant' spores, or in *Mycobacterium leprae* (which has never been artificially cultivated). The work of Heinmets and collaborators (e.g. Heinmets, Taylor & Lehman, 1953) on the resuscitation of 'dead' cells is a further reminder of the contingent nature of any possible definition.

I am indebted to my colleagues R. Elsworth, L. R. P. Meakin and R. C. Telling for information on samples taken from continuous cultures.

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EXPLANATION OF PLATE

- (a) Complete engraving tool.
- (b) Microscope objective with jet clipped on, for controlled drying.
- (c) Low-power view of a graticule lying on an agar surface. The small squares are about 100 μ . across.
- (d) High-power view of a single square of the graticule, with an inoculum of *Serratia marcescens*.

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The Nucleic Acids of *Sarcina lutea*

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SUMMARY: The deoxypentosenucleic acid (DNA) and pentosenucleic acid (PNA) were isolated from *Sarcina lutea* and their purine and pyrimidine contents determined. No differences were detected in composition of either the DNA or the PNA isolated from cultures grown at different times. The composition of the DNA from a streptomycin-resistant strain did not differ significantly from that of DNA from a normal streptomycin-sensitive strain, but the composition of the PNA from the resistant strain did differ significantly from that of PNA from the sensitive strain. The DNA contained a very high proportion of guanine and cytosine to adenine and thymine. In the PNA from both streptomycin-sensitive and resistant strains, guanine and cytosine predominated and the ratio of guanine + uracil to adenine + cytosine was nearly 1.

Considerable information has been obtained concerning the amount of nucleic acid present in bacteria at different stages of growth (Caldwell & Hinshelwood, 1950; Malmgren & Hedén, 1947; Fish, Asimor & Walker, 1950). In view of the active metabolic role of these substances and their demonstrated heterogeneity (Brown & Watson, 1953; Chargaff, Crampton & Lipshitz, 1953; Crosbie, Smellie & Davidson, 1953) it appears that changes in composition, as well as amount, may occur at different growth times, particularly with pentosenucleic acid (PNA). Also, considering the association of deoxypentosenucleic acid (DNA) with the transference of resistance to antibiotics (Hotchkiss, 1951; Alexander & Leidy, 1953) and of both PNA and DNA with the formation of certain enzyme systems (Gale & Folkes, 1955), a difference in composition of the nucleic acids from an antibiotic-resistant strain as compared with that of the nucleic acids from a sensitive strain, appears possible. To determine whether such differences occur in *Sarcina lutea*, the nucleic acids were isolated, separated into DNA and PNA and their purine and pyrimidine compositions determined. The compositions of nucleic acids from organisms grown for different times were compared, and those of nucleic acids from a normal streptomycin-sensitive strain compared with those of nucleic acids from a strain which had been trained to grow in the presence of streptomycin.

METHODS

The growth of Sarcina lutea. The organism was grown in liquid medium prepared as follows: a solution containing Oxoid bacteriological peptone (1%), Lab Lemco (1%) and sodium chloride (0.5%) was adjusted to pH 9 and autoclaved at 10 lb./sq.in. for 20 min. The resulting precipitate was filtered off, the filtrate adjusted to pH 7 and re-sterilized. Glucose (50%, w/v, aqueous solution) was added aseptically so that the final glucose concentration

was 1%. This medium gave a much better growth than that which had not been precipitated at pH 9. The organism was grown in 8 l. batches with aeration and vigorous stirring. For the preliminary work, growth was allowed to proceed at 37° for 48 hr. For comparison of the nucleic acids from organisms grown for different periods, organisms were harvested in the lag, logarithmic and stationary phases. Determination of the growth curves showed that the lag phase was 7–8 hr. and that the stationary phase began after about 90 hr.

Streptomycin-resistant strain of Sarcina lutea. The original strain of *Sarcina lutea* was susceptible to 1/16,000 streptomycin. By subcultivation in the presence of gradually increasing concentrations of the antibiotic, a strain was obtained which would grow in 1/500 streptomycin. The morphology of the organism was unchanged and it stained Gram-positive. It retained its resistance after numerous subcultures. For the isolation of the nucleic acids the organism was grown on medium with the same composition as that used for growth of the original streptomycin-sensitive strain.

Nitrogen was estimated by the method of Ma & Zuazaga (1942), and *phosphorus* by the method of Jones, Lee & Peacocke (1951).

Nucleic acid content. The nucleic acid content of *Sarcina lutea* was measured by the methods of Schmidt & Thannhauser (1945) and of Schneider (1945). In the latter method, DNA was measured by the diphenylamine colour reaction (Dische, 1930) and PNA by the phloroglucinol method of Euler & Hahn (1946).

Purine and pyrimidine content of the nucleic acids. The nucleic acids were analysed by acid hydrolysis followed by paper chromatography using the solvents of Laland, Overend & Webb (1952) and Wyatt (1951). For both PNA and DNA trifluoroacetic acid at 155° was used as the hydrolysing agent. The time of hydrolysis was 60 min. for DNA and 80 min. for PNA. This reagent was better than formic acid for the hydrolysis of DNA in that much less pressure was developed in the sealed tubes and the spots dried on the paper more quickly. It was better than perchloric acid for the hydrolysis of PNA because trifluoroacetic acid did not char the paper and effected complete hydrolysis of cytidylic acid without significant deamination (cf. Crosbie *et al.* 1953).

RESULTS

The nucleic acid content of Sarcina lutea

Sarcina lutea, which had been washed several times with distilled water and then freeze-dried, was used for the analysis. By the method of Schmidt & Thannhauser (1945) it was found that the organism contained 9.0% nucleic acid (assuming that the nucleic acid contained 8% phosphorus, the approximate value found for samples of *Sarcina lutea* DNA), of which 78% was PNA and 22% DNA. By the Schneider (1945) method, a total nucleic acid content of 8.5% was found, of which 81% was PNA and 19% DNA (using purified samples of *sarcina* PNA and DNA as standards). It was considered that both these methods were only approximate, but in view of the possible presence of other phosphorus-containing compounds in the cell which interfere with the

Schmidt & Thannhauser method (Mitchell & Moyle, 1954), the lower result, i.e. that obtained by the Schneider method, was taken as being the more accurate.

Isolation of the nucleic acids of Sarcina lutea

The nucleic acids were then isolated from a sample of the same batch of organisms (not freeze-dried) by the method already described (Jones, 1953; Dutta, Jones & Stacey, 1953). The organisms were disintegrated by shaking with ballotini glass balls at 0° for 3–4 hr., extracted with dilute sodium arsenate (0.01 M) at 0° and pH 7–7.5, then debris was removed by centrifuging and the nucleoproteins precipitated by the addition of cetrimide (cetyltrimethylammonium bromide, Cetavlon, I.C.I. Pharmaceuticals Ltd., Manchester). The nucleoprotein + cetrimide complex was purified by repeated reprecipitation from solution in M-sodium chloride, by dilution to 0.3 M-sodium chloride, with water. The nucleoproteins were then deproteinized by shaking repeatedly with chloroform and octanol (9:1; Sevag, 1934) and the nucleic acids further purified via their cetrimide salts and finally by precipitation with 20% (v/v) ethanol in water in the presence of 0.1 M-calcium chloride. By this method a yield of 6.5% total nucleic acids was obtained.

The loss of nucleic acid during the isolation may have been due to several factors, namely: (i) loss during cetrimide precipitation and subsequent reprecipitation; (ii) adsorption of nucleic acid on the gels formed during deproteinization by shaking with chloroform and octanol; (iii) loss during the final purification procedure for the removal of polysaccharides; (iv) incomplete disintegration and extraction of the organisms; (v) enzymic degradation. Certain of these possible sources of loss were examined.

Cetrimide precipitation of the nucleoproteins of Sarcina lutea

Sarcina lutea was disintegrated by shaking with glass beads, extracted with dilute sodium arsenate (pH 7–7.5) at 0° and the suspension centrifuged at 20,000 g until the supernatant liquid became almost clear (nucleoprotein solution). Samples of this solution were treated with various concentrations of cetrimide (0–2%), and the nucleic acids which remained in the supernatant liquids after centrifugation were precipitated with cold trichloroacetic acid and estimated by the method of Schmidt & Thannhauser.

For the precipitation of the nucleoproteins there was an optimum concentration of cetrimide (Table 1) and provided that this optimum was determined there would be little loss (c. 2%) of nucleic acid in the supernatant liquid. Losses during the purification of the nucleoprotein–cetrimide complex were negligible.

A major source of loss of nucleic acid was in the chloroform + protein gels formed during deproteinization. This nucleic acid was recovered by repeatedly extracting the gels with M-sodium chloride and purifying the recovered nucleic acid in the usual way. This brought the recovery of nucleic acid to about 92% of the total contained in the organisms. The other source of loss was during the final purification of the nucleic acids. By precipitating only

once with cetrimide and fractionating once with calcium chloride and 20% (v/v) ethanol in water this loss was kept below 10%. It was apparent that, with this organism, losses due to incomplete extraction and to enzyme activity were negligible.

Table 1. *The precipitation of the nucleoproteins of Sarcina lutea with cetrimide*

Cetrimide (%) ...	0	0.1	0.3	0.6	1.0	1.5	2.0
PNA phosphorus in solution ($\mu\text{g./ml.}$)	71.7	44.7	1.90*	3.42	12.5	14.2	14.3
DNA phosphorus in solution ($\mu\text{g./ml.}$)	23.2	16.7		1.37	3.60	4.92	5.15

* The precipitate was too small to be analysed for PNA and DNA, so only the total phosphorus was determined.

The nucleic acids were separated into DNA and PNA by fractionation with cetrimide, the PNA subsequently being purified by calcium precipitation and the DNA by adsorption with charcoal (Dutta *et al.* 1953). The recovery of nucleic acids for this stage was 78%, giving an overall recovery of 72%.

Composition of the nucleic acids

Using the above methods, the DNA and PNA were isolated from batches of organisms which had been grown for 6, 24, 48, 72, 96 and 144 hr., and from the strain which had been trained to be resistant to streptomycin, and analysed for their purine and pyrimidine bases. The results are recorded in Table 2.

Table 2. *Composition of the nucleic acids of Sarcina lutea*

Base	PNA		
	DNA	Normal strain	Streptomycin-resistant strain
		mole base/100 mole nucleotides	
Guanine	37.1	28.4	29.8
Cytosine	37.1	32.9	34.4
Adenine	13.4	16.7	15.7
Thymine	12.4	—	—
Uracil	—	22.0	20.2

For DNA, a recovery of about 3.8 moles of bases per 4 gram-atom of phosphorus was obtained. The composition of samples of DNA isolated from cultures grown for different times did not differ significantly (*c.* 1%) and was not greater than the difference between samples isolated from organisms grown for the same time. Also, no significant difference (*c.* 1%) was observed between the composition of the DNA from the normal streptomycin-sensitive strain and that of the DNA from the resistant strain. The bases were identified as

guanine, cytosine, adenine and thymine by their R_F values and ultraviolet absorption spectra. No 5-methylcytosine was detected.

For PNA, the presence of the usual four bases was confirmed and a recovery of about 4.0 mole of bases per 4 gram-atom of phosphorus obtained. The PNA from cells grown for different times did not show significant differences, but the PNA from the streptomycin-resistant strain had a different composition from that of PNA from the normal sensitive strain. Statistical analysis of the results showed that this difference was highly significant (e.g. for uracil, $t=5.10$; $P<0.001$).

DISCUSSION

For comparison of the compositions of nucleic acids isolated from different batches of organisms it was important that they should be obtained in good yield, so that any changes in composition would not be due to fractionation during isolation. It has often proved difficult to isolate nucleic acids quantitatively, particularly in the case of PNA (Allen, 1954). In the present work approximately 72% of the total nucleic acids was isolated. Although this was not as high as was desired, it was concluded that any changes in composition due to fractionation during isolation would be small since the isolation procedure was identical in each case.

The DNA, in common with those from many other bacteria, had a very high proportion of guanine and cytosine to adenine and thymine ($(G+C)/(A+T)=2.88$), in fact one of the highest yet recorded, and contained no 5-methylcytosine. The PNA had a larger amount of guanine and cytosine than adenine and uracil. Guanine and cytosine were not present in equimolecular proportions, neither were adenine and uracil, but the ratio of guanine + uracil to adenine + cytosine was very nearly equal to one (1.02). This PNA therefore showed one of the regularities pointed out by Elson & Chargaff (1954). It has been suggested by these authors that analysis of PNA *in situ* by mild alkaline hydrolysis would be expected to give more accurate results than analysis of PNA isolated by methods resulting in chemical and enzymic degradation. Such analyses, however, suffer from the disadvantage that the absence of interference by other cell constituents (e.g. DNA) cannot be certain in all organisms. Still more accurate results might be expected from the analysis of PNA which had been isolated by methods in which significant chemical and enzymic degradation had been eliminated. The present method of isolation appeared to approach fairly closely to this ideal in that all operations were carried out at 0° and near neutral pH; from the recovery of the nucleic acids it was apparent that enzymic degradation was only slight. There was no significant change in the composition of either DNA or PNA during growth of the cultures, a result similar to that obtained for the DNA of *Pseudomonas hydrophila* by Reddi (1954), and for the PNA of the sea-urchin *Paracentrotus lividus* by Elson, Gustafson & Chargaff (1954).

The inability to detect a difference in composition between the DNA from the streptomycin-sensitive strain of *Sarcina lutea* and that from the resistant strain does not imply that no differences exist. The fact that a transforming

principle, having the properties of DNA and capable of inducing streptomycin-resistance in sensitive strains, has been isolated from streptomycin-resistant strains of *Haemophilus influenzae* (Alexander & Leidy, 1953) indicates that the DNA from resistant strains does differ from that from sensitive strains. In our work with the DNA from *Sarcina lutea*, however, such a difference may have had no effect on the proportions of the bases present, or the difference in proportions may have been too small to detect by the present methods. It has been shown that in *Staphylococcus aureus* PNA is associated with the production of certain enzymes (Gale & Folkes, 1955). Since streptomycin-resistant strains would use a number of different enzymes from the normal strain, this may be reflected in a change in the overall composition of the PNA as has been found in this work with *Sarcina lutea*. This change in composition may have been due either to the synthesis of new PNA associated with new enzyme systems necessary for growth in the presence of streptomycin, or merely to a change in the proportions of the PNA already present in the normal streptomycin-sensitive strain. An increase in the amount of PNA present in streptomycin-resistant strains of *Staphylococcus aureus* and *Haemophilus pertussis* compared with that in sensitive strains, has been recorded (Smolens & Vogt, 1953; Beljanski, 1953).

In the PNA of streptomycin-resistant *Sarcina lutea* there was an increase in the proportion of guanine and cytosine, and a corresponding decrease in adenine and uracil as compared with PNA from the normal sensitive strain. This result gives some support to the hypothesis (Elson & Chargaff, 1954) that in part of the PNA, these bases may be associated in a manner similar to those in DNA (Watson & Crick, 1953). The ratio guanine+uracil to adenine+cytosine was almost unity (0.998), so that this PNA also showed the same regularity as that from the streptomycin-sensitive strain and those from other organisms.

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The Induction of Bacteriophage in Staphylococci

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SUMMARY: The induction properties to ultraviolet light of some of the staphylococcal phages of the National Collection of Type Cultures (Colindale, London) and their propagating strains were studied. There was considerable variation in response, but in all cases phage titres rose. Bacterial opacity was an unsatisfactory indicator of induction and on some occasions no lysis was detected.

The observation that some bacteria undergo induction with a number of agents, both physical and chemical, has been studied intensively in the last few years. In 1953, Lwoff published an extensive review of the field, including a useful series of definitions which will be used in this paper. At about the same time the work on induction in staphylococci was reviewed by Welsch, Cavallo & Cantelmo (1953). The present paper is a report of some work designed to study the induction properties of the National Collection of Type Cultures (NCTC) staphylococcal typing phages with ultraviolet light. This study of a group of readily available phages was undertaken to see whether it was possible to reproduce the findings of other workers with temperate phages. Most of the systematic work has been concerned with the phages of the Gram-negative rods, especially *Escherichia coli* strain K 12.

Before this could be achieved it was essential that the phage should lysogenize a suitable test staphylococcus, which could then be irradiated with known doses of ultraviolet light. Since all the typing phages have a corresponding NCTC propagating staphylococcus it was decided to use these staphylococci as the test organisms. The effect of irradiation on these staphylococci had to be studied first to see whether they were lysogenic so that the change caused by the addition of the typing phage could be recognized. As will be seen from the results in Table 1, all NCTC staphylococci were found to show some signs of induction, which is not surprising since staphylococci are frequently found to be lysogenic, often with more than one phage. The interpretation of the results therefore is not as simple as one might wish, as in every case the induction pattern represents the natural pattern of the test organism mixed with that of the phage studied. This state of affairs may be fairly common since indicator cells are lysogenic more often than is commonly realized, while studies of naturally lysogenic strains are often confused by the presence of a second phage which may not be recognized for some time (Welsch *et al.* 1953).

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METHODS

Considerable difficulties were experienced in preparing a suitable standard medium for counting phage and bacterial growth. Finally the following medium (PYLP) was used, enough of each constituent being purchased to allow the work to be completed without change. Peptone (Evans), 10 g.; Yeastrel (Brewers Food Supply Co. Ltd., Edinburgh 3), 3 g.; Lab Lemco, 5 g.; Pronutrin (Herts Pharmaceuticals Ltd., Welwyn Garden City), 10 g.; sodium chloride (AR), 5 g.; distilled water to 1 l. This mixture was adjusted to pH 7.4, and autoclaved at 10 lb./sq.in. for 10 min. Difco agar to 2% (w/v) was added to convert to a solid medium as required. All phage dilutions and all irradiations of cultures were done in the 56 phosphate buffer of Monod, Cohen-Bazire & Cohn (1951).

The cultures were irradiated at 1 m. distance from a 30 W. G.E.C. germicidal tube. The output of this tube was measured at the target by constructing a killing curve for T2 bacteriophage and comparing the result with the data given by Latarjet, Morenne & Berger (1953). From this comparison a value of 4.5 ergs/sq.mm./sec. was obtained. This is lower than the value obtained earlier with the same lamp (10 ergs), but the experiments were repeated on occasions throughout the work and always yielded values of *c.* 4.5 ergs. It seems possible that the bactericidal potency of the lamp decreased with time from its initial value.

Procedure for induction. Cultures were prepared for induction by growing them with continuous shaking in 10 ml. PYLP medium at 37° for 3 hr. They were then centrifuged, the supernatant removed for free phage counts and the deposit resuspended in an equal volume of cold 56 buffer. With all the staphylococci used the bacterial opacity and bacterial counts at this point were similar.

All growth curves were followed by the change in opacity as measured by a Spekker photoelectric absorptiometer (Hilger and Watts Ltd.). To facilitate these measurements, and at the same time allow continuous agitation during growth, the cultures were kept in special tubes. These tubes consisted of precision bore glass (Chance precision glass tubing) internal diameter 15 mm. and 6 in. long; one end was sealed and the barrel was bent at right angles in the middle. This angle tube held 10 ml. of medium in its lower part, and fitted into a specially constructed carrier in the Spekker absorptiometer, thus allowing opacity readings to be made rapidly. In order to incubate the culture the tubes were clipped into a water-bath with the lower end parallel with the surface of the water. Gentle rocking of the tube by mechanical means gave excellent aeration and by this means cultures were kept in the log phase.

In an experiment 8 ml. of PYLP was added to each of four tubes. The 10 ml. staphylococcal culture was centrifuged and resuspended in 10 ml. 56 buffer and 2 ml. of this suspension added to the first tube. The 8 ml. remaining were poured into a 10 cm. Petri dish. This culture was then irradiated during gentle rocking, 2 ml. samples being removed at intervals and added to the other

tubes. From then on the tubes were kept in the dark, except during opacity readings, to prevent photo-reactivation. Opacity readings were made every 30 min. and 1 ml. samples were taken at hourly intervals for the free phage count.

Duration of irradiation. After various trials it was found that the most convenient times for irradiation were 30, 60 and 90 sec. In all cases longer periods were tried when a culture was non-inducible, but the longer irradiation never succeeded in producing a change in behaviour. In most cases times of 2 min. or more appeared to kill so many staphylococci that very little growth took place. The cultures after irradiation were observed for 3 hr. which was sufficient time to cover the whole period of induction and phage release.

Phage titration. Counts were made on supernatant fluids only, the samples being centrifuged at 3000 r.p.m. for 20 min. Tenfold serial dilutions were made and a wire loop of 3 mm. internal diameter was charged with the dilution and used to spread the fluid over a segment of a plate previously inoculated with the indicator strain of staphylococcus. The loop delivered 0.01 ml. reasonably accurately. Loops were calibrated and checked at intervals by counting samples of phage T2 in parallel by this method and by the agar layer method (Adams, 1950).

Counts with a plate microscope were made on that dilution which gave between 20 and 200 plaques. The number of infective phage particles/ml. culture was then calculated. The initial phage count was made on the supernatant fluid of the pre-irradiated culture, and hence the values obtained were five times too high when compared with the rest of the phage counts which were made on the dilution tubes; this has been corrected in Table 1.

Lysogenic strains. The 13 NCTC propagating strains were tested for lysogenicity by the cross-culture method of Fisk (1942) using over 100 different strains of coagulase-positive staphylococci as indicators. All but 2 of the 13 strains were found to be carrying phage; no attempt was made to see whether there was more than one phage present. When several indicators were sensitive to a phage the indicator which gave the clearest plaques was chosen, for ease of counting.

In order to render a NCTC staphylococcus lysogenic with its NCTC phage, they were grown together until a stable culture was obtained. Before being used in these experiments all cultures were subjected to three tests: (a) the culture had to be stable, i.e. no signs of erosion or phage action on the isolated colonies; (b) every colony had to be lysogenic, as tested by the replica plate method of Lederberg & Lederberg (1952); (c) the culture then had to be immune to the carried phage. Several combinations of staphylococcus and phage had to be discarded because of failure to satisfy these criteria of lysogenicity.

Terminology. Where possible the NCTC numbers are used throughout. When a staphylococcus was lysogenized by a phage the new culture was given a number made of the bacterial number followed by the phage number, e.g. 8321/8410 is staphylococcus 8321 lysogenized by phage 8410. The indicators for the phages carried by the NCTC staphylococcus strains are given their

Table 1. Induction patterns of some NCTC staphylococci and the corresponding NCTC typing phages

Strains before lysogenizing					Lysogenized strains				
Culture	Indicator staphylococcus	Non-irradiated phage titre	Irradiated		Indicator staphylococcus	Non-irradiated phage titre	Irradiated		Effect of added phage on induction
			Phage titre	Degree of lysis			Phage titre	Degree of lysis	
8321	27	10 ⁵	10 ⁹	Moderate	8321/8410	10 ⁵	10 ¹⁰	Moderate	None
8325	H	10 ⁵	10 ⁸	Moderate	8325/8409	10 ⁴	10 ⁷	Marked	Increased
8345	None	Not tested	Not tested	Slight	8345/8415	10 ⁵	10 ⁸	None	Decreased
8349	86	10 ⁵	10 ⁷	Moderate	8349/8416	10 ⁵	10 ⁸	Moderate	None
8352	51	10 ⁵	10 ¹⁰	None	8352/8425	10 ⁵	10 ⁹	Slight	Increased
8353	H	10 ⁴	10 ⁷	Marked	8353/8417	10 ⁵	10 ⁹	Marked	None
8355	29	10 ⁵	10 ⁸	Moderate	8355/8421	10 ⁵	10 ⁷	Slight	Decreased
8356	60	10 ⁵	10 ⁸	Moderate	8356/8428	10 ⁴	10 ¹⁰	Moderate	None
8357	16	10 ⁵	10 ⁹	Slight	8357/8418	10 ⁵	10 ⁸	Slight	None
8358	86	10 ⁴	10 ⁸	Slight	8358/8429	10 ⁵	10 ⁷	Slight	None
8363	H	10 ⁵	10 ⁸	Slight	8363/8290	10 ⁴	10 ⁸	Marked	Increased
—	—	—	—	—	8363/8420	10 ⁴	10 ⁸	Slight	None
8508	None	Not tested	Not tested	Slight	8508/8405	10 ⁵	10 ¹⁰	Slight	None
8509	98	10 ⁶	10 ⁷	None	8509/8403	10 ⁵	10 ⁸	None	None

local number or letter, e.g. 8321 carries a phage which will give plaques on staphylococcus no. 27.

When counting the induced phage, phage 8321 was counted on staphylococcus 27, while phage 8321/8410 was counted on staphylococcus 8321. This latter method ensured that only phage 8410 was being counted unless the phage of 8321 gave a virulent mutant, which seemed unlikely in any large numbers.

It would have been interesting if, when phage 8321/8410 was induced, it had been possible to count phage 8410 and the phage of 8321 separately. Unfortunately, the NCTC phage always made plaques on the indicator strain; for reasons that are to be discussed later it never proved satisfactory to try to separate the two counts.

RESULTS

Some combinations failed to give rise to stable lysogenic systems because the cultures were unstable (8317/8407, 8354/8426 and 8511/8406), or the culture yielded mainly G variants (8319/8408), or despite several attempts the phage was gradually lost (8331/8413, 8341/8414, 8507/8401, and 8508/8287). In a few cases the phage made such poor plaques on its indicator staphylococcus that the combination was discarded (8355/8419 and 8508/8402).

There was considerable difficulty in obtaining reproducible results. Despite the use of cultures in the same stage of growth in the same medium, the degree of induction and the phage titre were liable to unaccountable variations. Each experiment was therefore repeated on several occasions and as all strains were freeze-dried the repetitions were made over a period of months. When this was done the strains showed a standard behaviour from which they departed only occasionally. The results reported here were obtained at least three times and are regarded as the average. By using the NCTC collection it was possible to repeat with fresh cultures any experiment which seemed unusual, without fear of having a contaminated stock.

The results are shown in Table 1. The degree of lysis following irradiation has been divided into four grades. (There was, of course, some slowing in growth rate of normal bacteria following irradiation, and this was allowed for when inspecting the curve).

These degrees of lysis were:

- | | |
|-----------------|--|
| <i>None</i> | Where the opacity curves appeared similar to those of non-lysogenic bacteria. |
| <i>Slight</i> | Where the opacity curve either became level or dipped slightly at some point. |
| <i>Moderate</i> | Where the opacity after increasing showed a fall to the initial level. |
| <i>Marked</i> | Where the degree of clearing was such that at some point the opacity was much less than the initial value. |

Phage counts were done at hourly intervals, and by the end of the first hour showed a considerable rise when compared with the initial count. This result

agrees with the findings of Welsch *et al.* (1953), who measured the latent period in staphylococci. The highest average counts usually found by the second or third hour are given in every case. The initial counts are the average of those of the supernatant fluid of the initial culture. All counts are of course related to the number of staphylococci present, but as mentioned earlier the cultures were very similar in numbers and growth rate and so were comparable.

Some interesting points may be noted (see Table 1). In two strains the addition of another phage appeared to make the staphylococcus less inducible. This was observed on several occasions in parallel and occurred constantly. The two NCTC staphylococci for which no indicator was found showed some lysis on irradiation. This finding suggests that in fact all the NCTC strains are lysogenic, and it also illustrates the difficulty of eliminating any possibility of a strain being lysogenic.

Following irradiation the phage titres rose in every case, even where there was no lysis, much more than can be explained on the basis of an increase in bacterial growth, since the phage counts in the control tube kept in step with the increase in bacterial numbers. The increase in titre varied between a hundred-fold and a million-fold, and there was no correlation between increase in phage titre and degree of bacterial lysis.

DISCUSSION

It is apparent that the study of staphylococci and their lysogenic phages presents many technical difficulties. The greatest difficulty is to find a non-lysogenic strain which can be infected with the NCTC phages. Hence all the curves are composed of the original bacterial induction patterns plus the effect of the added phage. It seemed that in two instances the addition of another prophage rendered the bacteria less inducible. One explanation that was considered was the substitution of the original prophage by the second phage (Bertani, 1953). Unfortunately, the NCTC phages used always made plaques on the indicators which were suitable for testing the phages carried by the NCTC staphylococci. Some experiments were done with the purpose of rendering the indicator lysogenic and hence immune to the NCTC phage. While it was successful in making the strain immune it appeared to produce a less efficient indicator for the phage carried by the NCTC staphylococcus. With this less efficient indicator there was no evidence of substitution in the less inducible strain.

Induction in the sense of bacterial lysis is usually coincidental with phage increase. In this work there were several examples where phage titres increased rapidly to high values with little or no bacterial lysis. Somewhat similar findings can be found in some of the data of Welsch *et al.* (1953) and by Goffart-Roskam (1952). The most likely explanation of this discrepancy is given by Farrant & Rountree (1953), who studied the lysis of staphylococci by phage A3. They found that after phage liberation the cell membrane remained as a light-scattering object so that the increase in phage titre and the decrease in bacterial opacity did not change proportionately.

In the example where one staphylococcus (8363) was lysogenized by two phages (8290 and 8420), it will be seen that the induction patterns differ. This is similar to the finding of Jacob (1952) and other workers that inducibility is mainly a property of the prophage. However, the results here differ from another conclusion in the paper of Jacob, namely that the more inducible phages have the highest free phage counts in the non-induced culture. There is, however, the possibility that the phage counts are influenced by the degree of adsorption of each phage by its host cells or by bacterial debris.

Further work is proceeding on the cultures which were unstable. It has been found that in at least one case instability is due to the appearance of a more virulent phage which attacks the cocci which are lysogenic with the temperate strain. This regularly gives rise to unstable colonies.

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The Induction of the Lytic Cycle in Lysogenic Bacteria by Phagolessin A 58

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SUMMARY: Phagolessin A 58, an antibiotic having antiphage activity, was found to have an action which induced the lytic cycle in some strains of lysogenic bacteria. Four different lysogenic organisms were tested: *Bacillus megaterium* 899; *Escherichia coli* FCb; *E. coli* Temple; *E. coli* Lampert. Two of the organisms were induced by phagolessin A 58 and two were not. There appeared to be a relationship between the susceptibility of a given strain to induction by ultraviolet irradiation and to induction by phagolessin A 58. The four different phages carried by the respective lysogenic strains were inactivated to a greater or lesser extent by phagolessin A 58, but there was no correlation between such sensitivity on the part of the carried phage and inducibility of the particular lysogenic strain. Serial transfers of the four lysogenic strains in broth containing phagolessin A 58 were made in an attempt to produce non-lysogenic, phage-sensitive mutants. With only one organism, *Bacillus megaterium* 899, did this treatment result in the selection of such a mutant.

Some of the chemical and biological properties of phagolessin A 58 were described in previous publications (Asheshov, Strelitz & Hall, 1952; Hall & Asheshov, 1953), where it was shown that it caused an apparently irreversible inactivation of free phage particles after a relatively short contact. Not all of the phage strains tested were sensitive to its action, some 10 out of 60 strains being completely resistant. The phagocidal action could be blocked by the addition of deoxyribose nucleic acid. The present investigation was undertaken to determine whether phagolessin A 58 could also inactivate the intracellular phage carried by lysogenic bacteria—the 'prophage' of Lwoff (Lwoff & Gutmann, 1950)—and, in effect, 'cure' lysogenic cells. It was found that the main action of phagolessin A 58 on lysogenic systems was an inducing one—i.e. it caused lysogenic cells to enter the lytic cycle, lyse and release their phage.

METHODS

Phagolessin A 58: The preparations of phagolessin A 58 used throughout this investigation were methanol solutions of lyophilized concentrates and contained small amounts of impurities. Concentrations, expressed as dilution units/ml., were based on the results of weekly serial dilutions against cholera phage C, the phage chosen as standard for this antibiotic.

Media. Nutrient broth was prepared as follows: papain-digest of horse meat (Asheshov, 1941), 400 ml.; yeast extract (Difco), 2.5 g.; trace element solution, 1 ml.; tap water, 600 ml. The pH value was adjusted to 7.2.

Solid medium was prepared by adding 7.5 g. New Zealand agar (Davis) to 1 l. of nutrient broth.

Streptomycin plates, used in some experiments, were prepared as above, with the further addition of 1000 units of streptomycin/ml.

Buffers. Phosphate buffer: a concentrated solution containing 87 g. Na_2HPO_4 (anhydrous Sorensen) and 53 g. KH_2PO_4 (anhydrous Sorensen)/l. distilled water was used to prepare solutions as follows. M/15 buffer for UV radiation experiments; the concentrated buffer solution was diluted 1/15 in distilled water. NaCl was added to a final concentration of 0.5% (w/v) and MgSO_4 to a final concentration of M^{-3} . For M/50 buffer the concentrated buffer solution was diluted 1/50 in distilled water. Unless otherwise noted, there was no further addition. In all cases the final pH value was adjusted to 7.0.

Lysogenic cultures and indicator strains. Four different lysogenic cultures were studied. They were *Bacillus megaterium* strain 899, first described by Den Dooren de Jong (1930), and three lysogenic strains of *Escherichia coli*—FCb, Temple and Lampert. The culture of *Bacillus megaterium* 899 and its indicator strain, *B. megaterium* PR3E, were kindly supplied by Professor P. B. Cowles. *Escherichia coli* FCb was obtained from Dr N. A. Boulgakov, *E. coli* Temple from Dr M. Rakieten. *E. coli* Lampert was one of the host strains in the J. Ward MacNeil collection. The indicator strain used with *E. coli* FCb was *Shigella dysenteriae* Y6R: a strain of *S. flexneri* var. Newcastle was used as indicator for *E. coli* Temple and *E. coli* Lampert. All cultures were maintained on agar slants at 10°.

Production of cell-free phage filtrates. The techniques used varied with the lysogenic strain. With *Bacillus megaterium* 899 and *Escherichia coli* Lampert, high titre stocks were best obtained by filtering a 24 hr. broth culture of the lysogenic strain. When this procedure was used with *E. coli* Temple or *E. coli* FCb, the titres of the filtrates were rather low, and better results were obtained by growing the phage on the appropriate indicator strains. Pyrex sintered glass filters (UF) were used as a routine. Phage filtrates were stored at 10°. They were all rather unstable and it was necessary to prepare fresh filtrates at frequent intervals. The phages carried by the four lysogenic strains will be referred to by the name of the strain carrying them: 899, FCb, Temple, Lampert.

Use of streptomycin-resistant indicator strains in assaying phage. Bertani (1951) described a technique for assaying phage in a mixture of lysogenic cells and free phage which proved useful in the present study. According to this method, assays for free phage particles are made on streptomycin plates inoculated with a streptomycin-resistant mutant of the indicator strain. Lysogenic organisms are inhibited by the streptomycin and so do not form colonies; clearings produced by free phage particles can then be seen easily. This method was used in assaying free phage in the three lysogenic strains of *Escherichia coli* where, under normal conditions of growth, the cell concentration greatly exceeded the free phage concentration. It was not found necessary to use this method in assaying free phage in cultures of *Bacillus megaterium* 899 since, in this culture, the free phage concentration was higher than the cell concentration. The phages carried by the three lysogenic strains of *Escherichia*

coli were resistant to the concentration of streptomycin used, and free phage particles plated equally well on plain or on streptomycin plates. The three phages also showed the same efficiency of plating on plain agar regardless of whether they were plated on the streptomycin-sensitive parent strain or on the streptomycin-resistant mutant of the indicator strain.

Method used in assaying bacteria and phage. The loop dilution method of Asheshov & Heagy (1951) was used in assaying bacteria and phage. Phage assays were made on plates previously flooded with the appropriate indicator strain. All counts represent the mean of duplicate platings.

Ultraviolet radiation. Lwoff, Simonovitch & Kjeldgaard (1950) discovered that a relatively short exposure to ultraviolet (UV) radiation induced some lysogenic cultures to enter the lytic cycle and, after a definite latent period, to lyse and release a normal burst of newly formed phage particles. The technique used in the present study was as follows. Actively growing organisms in nutrient broth were centrifuged down, washed to remove free phage particles, and resuspended in M/15 phosphate buffer. Five ml. of the buffer suspension were placed in a sterile Petri dish and irradiated for varying periods of time. Following irradiation the organisms were diluted 1/10,000 in nutrient broth and incubated at 37°. After 30–45 min. of incubation, platings were made to count colony-formers and clearing-producers. The UV source was a high-pressure mercury arc lamp, and irradiation was done at a distance of 40 cm., the intensity of irradiation at this distance being such that phage T2 was 99% inactivated after 7 sec. of exposure.

RESULTS

Characteristics of the four lysogenic organisms

Bacillus megaterium 899. This culture has been described in detail by a number of workers, therefore only a brief summary of our results will be given here. The multiplication of organisms and production of phage during growth in nutrient broth at 35° is represented in Fig. 1. During the period of exponential multiplication of bacilli the phage production ran approximately parallel, with a constant ratio of phage to organisms of 6:1. Free phage particles of phage 899 were readsorbed by the lysogenic bacilli and a proportion was therefore 'lost' as infective centres (Lwoff & Gutmann, 1950).

Irradiation of the culture by UV light causes induction with lysis of the cells and release of free phage particles (Lwoff *et al.* 1950). The amount of induction obtained by the technique described above is expressed as a function of UV dose in Fig. 2. Maximum induction was produced after exposure for 60 sec. Induced cells began to lyse 65 min. after irradiation, with release of c. 100 phage particles/induced organism.

When non-induced organisms of this culture were plated on the indicator strain, each organism gave rise to a colony which was surrounded by a zone of lysis. Induced organisms, on the other hand, produced clearings on the indicator strain which were identical with those produced by free phage particles.

Escherichia coli FCb. The multiplication of organisms and production of free

phage by this culture, grown in nutrient broth at 35°, is shown in Fig. 3. Free phage increased logarithmically at a rate equal to that of the organisms, with a constant ratio of free phage to cells of 1:100. Free phage particles were not adsorbed by lysogenic organisms at an appreciable rate.

This culture could be induced by UV radiation. In Fig. 4, the amount of induction is plotted as a function of UV dose. Maximum induction was obtained after exposure for 10–15 sec. Induced organisms began to lyse 120–130 min. after irradiation, and the average burst size was approximately 250 phage particles/induced organism.

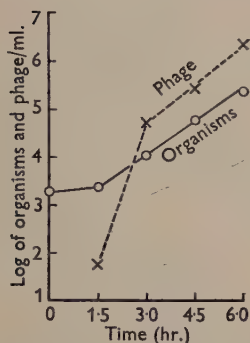


Fig. 1

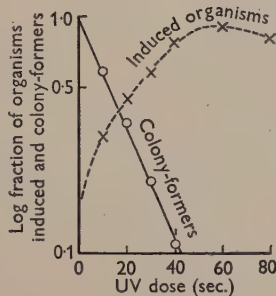


Fig. 2

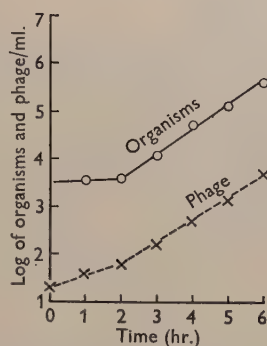


Fig. 3

Fig. 1. Multiplication of organisms and production of phage by lysogenic *Bacillus megaterium* 899 in nutrient broth at 35°.

Fig. 2. Induction of lysogenic *Bacillus megaterium* 899 by UV radiation, expressed as a function of dosage. The log of the fraction of colony-formers and organisms induced is plotted against UV dose (sec.)

Fig. 3. Multiplication of organisms and production of phage in lysogenic *Escherichia coli* FCb in nutrient broth at 35°.

When non-induced organisms of this culture were plated on the indicator strain, less than 1% gave rise to colonies surrounded by a very narrow zone of lysis, the majority showed no evidence of lysogenicity. Induced organisms failed to form a colony and produced clearings on the indicator strain. Clearings produced by free phage particles of phage FCb were clear-cut and did not show any resistant growth, and there was considerable variation in the size of the clearings. On the other hand, clearings produced by UV-induced organisms before lysis were larger and more regular in size and outline than those which developed from free phage particles. Similar results were reported by Weigle & Delbrück (1951) for the lysogenic strain of *Escherichia coli*, K-12.

Escherichia coli Temple. Under normal conditions of growth very few organisms of *E. coli* Temple appeared to undergo spontaneous lysis. The growth curve and the free phage concentration are shown in Fig. 5. Nutrient broth was the medium used in this experiment and incubation was at 35°. Phage production ran parallel to multiplication of organisms, with a constant ratio of phage to organisms of 1:300. *E. coli* Temple was not induced by

exposure to UV radiation. Such treatment resulted in a loss of viable organisms at an exponential rate without any release of phage (Fig. 6).

Organisms of this culture, plated on the indicator strain, gave no evidence of lysogenicity. Clearings of free particles of phage Temple were of medium size with irregular edges and showed a central area of secondary growth which was found to consist of the indicator strain lysogenized by phage Temple. Free particles of phage Temple were not adsorbed by the lysogenic cells at a measurable rate.

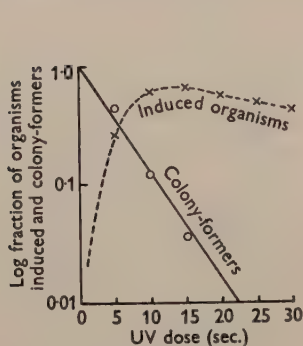


Fig. 4

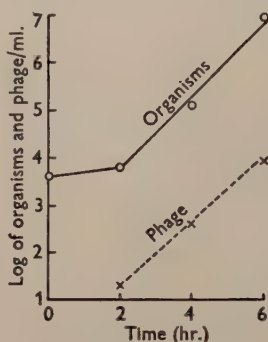


Fig. 5

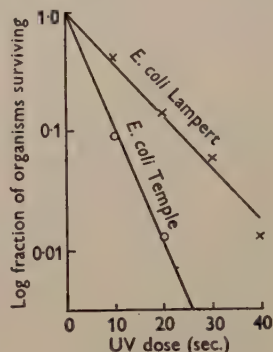


Fig. 6

Fig. 4. Induction of *Escherichia coli* FCb by UV radiation expressed as a function of dosage. The log of the fraction of colony-formers and organisms induced is plotted against UV dose (sec.).

Fig. 5. Multiplication of organisms and production of phage by lysogenic *Escherichia coli* Temple in nutrient broth at 35°.

Fig. 6. Inactivation of *Escherichia coli* strains Temple and Lampert following UV radiation. There were no induced organisms. The log of the fraction of cells able to form colonies is plotted against UV dose (sec.).

Escherichia coli Lampert. This culture resembled *E. coli* Temple in a number of respects. The amount of free phage released into the medium during growth in nutrient broth at 35° was very small (Fig. 7). It was characteristic of this culture that phage production ran parallel to multiplication of organisms only at the beginning of the logarithmic growth phase. At that time the ratio of phage particles to organisms was approximately 1:500. Subsequently very little free phage was produced. Experiments showed that phage Lampert was not adsorbed by lysogenic organisms at a measurable rate, so that the low concentration of free phage particles during the latter part of the growth curve could not be accounted for by assuming that the free phage particles were being readsorbed on to lysogenic organisms and so lost. The results, therefore, suggests that the probability of an organism undergoing spontaneous lysis is greater during the early part of the logarithmic growth phase than at any other time during the growth period.

The culture was not inducible by UV radiation, the only effect of such treatment being an inactivation of viable organisms without any release of active phage (Fig. 6).

Escherichia coli Lampert plated on the indicator strain showed no evidence of lysogenicity. Clearings of phage Lampert were extremely small and were quite clear and apparently free from resistant growth.

Action of phagolessin A58 on free phage particles

Free particles of the four carried phages were inactivated to a greater or lesser extent following exposure to phagolessin A58. The rate of inactivation of each phage was studied over a period of time at one concentration of the

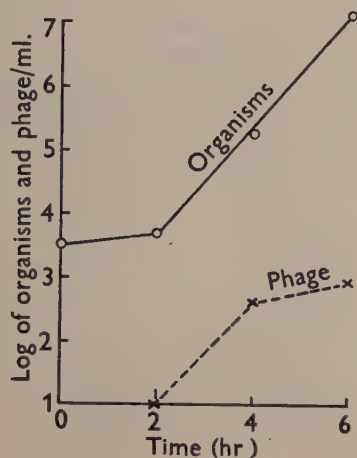


Fig. 7

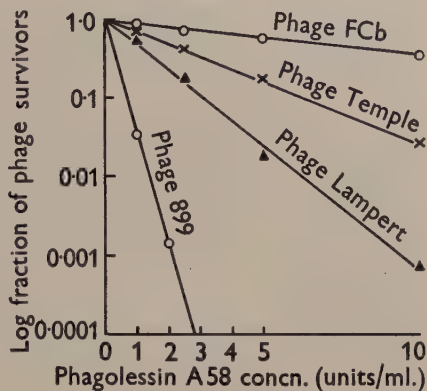


Fig. 8

Fig. 7. Multiplication of organisms and production of phage in lysogenic *Escherichia coli* Lampert in nutrient broth at 35°.

Fig. 8. Inactivation of the four phages (899, FCb, Temple, Lampert) by exposure to different concentrations of phagolessin A58. Each phage was diluted in nutrient broth and mixed with varying concentrations of antibiotic at 35° for 10 min. Following this they were rapidly diluted and plated to count survivors. The log of the fraction of survivors is plotted against antibiotic concentration (units/ml.).

antibiotic. In these experiments the suspending medium was nutrient broth and inactivation was carried out at 35°. The rate of inactivation was exponential with velocity constants per min. at 1 unit/ml. of phagolessin A58 of 0.14 for phage 899, 0.0048 for phage FCb, 0.016 for phage Temple, 0.030 for phage Lampert. The rate of inactivation varied directly with the concentration of phagolessin A58 used (Fig. 8).

Action of phagolessin A58 on lysogenic cells

The majority of experiments were conducted with *Escherichia coli* FCb, and only these results will be reported in any detail. *E. coli* FCb was exposed to varying concentrations of phagolessin A58 in nutrient broth at 37°. The starting inoculum, which consisted of $c. 4 \times 10^3$ organisms/ml., was prepared by diluting a suspension of organisms from an 18 hr. agar slope. The cells were in the lag phase of growth. Three different concentrations of phagolessin A58 were used (10, 20 and 30 units/ml.) and colony counts and phage counts were made

at hourly intervals for 3 hr. Phage counts were made on both plain and on streptomycin plates, both inoculated with the streptomycin-resistant mutant of the indicator strain. The results are summarized in Table 1.

Table 1. *Effect of phagolessin A58 on cell growth and phage production in Escherichia coli FCb*

Organisms in nutrient broth at 37°, were exposed to 0, 10, 20 and 30 units phagolessin A58/ml. Counts of colony-formers and clearings were made at hourly intervals for 3 hr. Phage counts were made on plain and streptomycin agar, both inoculated with the streptomycin-resistant mutant of the indicator strain.

Period of incubation (hr.)	Concn. of A58 (units/ml.)	Colony count (no./ml.)	Phage count (clearings/ml.)	
			Plain agar	Streptomycin agar
1	0	4.6×10^3	2.0×10^1	2.0×10^1
	10	3.4×10^3	1.6×10^2	$< 2.0 \times 10^1$
	20	1.5×10^3	1.4×10^3	$< 2.0 \times 10^1$
	30	4.8×10^2	1.9×10^3	$< 2.0 \times 10^1$
2	0	5.0×10^3	2.0×10^1	2.0×10^1
	10	2.1×10^3	1.2×10^3	$< 2.0 \times 10^1$
	20	2.6×10^2	1.9×10^3	$< 2.0 \times 10^1$
	30	$< 2.0 \times 10^1$	2.2×10^3	$< 2.0 \times 10^1$
3	0	6.6×10^2	8.0×10^1	8.0×10^1
	10	1.0×10^3	8.8×10^2	$< 2.0 \times 10^1$
	20	$< 2.0 \times 10^1$	1.7×10^3	$< 2.0 \times 10^1$
	30	$< 2.0 \times 10^1$	1.2×10^3	$< 2.0 \times 10^1$

Judging by the loss in colony-formers in those tubes which contained phagolessin A58, the antibiotic appeared to exert a strong bactericidal action. The marked increase in phage concentration caused by the antibiotic suggested, however, that the action was an inducing one—i.e. that lysogenic cells were being induced to enter the lytic cycle, and after a period of intracellular phage growth, to lyse and release their phage. Several observations showed that phage clearings which were present on plain plates but not on streptomycin plates were due, not to free phage particles, but to induced organisms which were intact at the time of plating but which lysed after plating and so did not form a colony. In the first place, phage FCb is fairly sensitive to inactivation by phagolessin A58 and one would expect few, if any, of the free phage particles to survive any of the three concentrations used. In the second place, the clearings were larger and more regular in size and shape than was usually the case with free phage particles. They resembled the clearings produced by UV-induced cells. Also, the discrepancy between the phage counts depending on whether platings were made on plain or on streptomycin plates was added evidence that such clearings did not arise from free phage particles. Free phage particles plate equally well on plain or on streptomycin plates, and it seemed probable that induced organisms would be inhibited by streptomycin and would fail to lyse and release their phage.

As a test of the hypothesis that organisms in which induction had been initiated by phagolessin A58, were, in fact, inhibited by streptomycin, it was

decided to see whether the latter had a similar offset on organisms induced by UV radiation. *Escherichia coli* FCb was irradiated in buffer for 15 sec., diluted into broth and incubated at 37°. Phage counts were made at intervals on plain and on streptomycin plates. The results, presented in Fig. 9, showed that up to 90 min. after induction (*c.* three-quarters of the latent period) induced organisms were unable to produce clearings in the presence of streptomycin. After 90 min. the number of organisms which produced clearings increased rapidly until, at 120 min., when lysis began, virtually all of the induced organisms produced clearings on the streptomycin plates. There is, therefore, a 'streptomycin-sensitive' phase followed by a 'streptomycin-resistant' phase. It is possible that the phase of streptomycin-resistance is correlated with the appearance of mature phage particles inside the cell. On the basis of these results we concluded that the action phagolessin A58 on *E. coli* FCb is not a direct bactericidal one but an inducing one.

Induction of Escherichia coli FCb by phagolessin A58

In order to demonstrate induction more clearly a technique was adopted similar to that described by Williams Smith (1953) for demonstrating induction of lysogenic *Salmonella* strains by nitrogen mustard. Suspensions of *Escherichia coli* FCb in the exponential growth phase were centrifuged, washed free of phage particles and resuspended in M/50 phosphate buffer. The suspension was divided into two parts and 15 units phagolessin A58/ml. were added to one part, the other serving as a control. The suspensions were incubated at 37°, and at regular intervals samples were removed, diluted 1/20,000 in nutrient broth and plated to count colony-formers and induced organisms. Fig. 10 shows the results of such an experiment where the fraction of colony-formers and induced organisms is plotted as a function of time of exposure to phagolessin A58. The picture is similar to that obtained with UV induction (Fig. 4) and shows that maximum induction occurred between 15 and 30 min.

In an extension of this experiment, organisms, induced by exposure to 15 units phagolessin A58/ml. for 20 min., were diluted in nutrient broth, incubated at 37°, and plated at regular intervals in order to determine the length of the latent period and the average burst size/induced organism. Lysis began 90–100 min. after induction, with an average burst size of *c.* 200–250 phage particles/induced organism. Organisms induced by phagolessin A58, therefore, had the same average burst size as UV-induced organisms, but the organisms lysed some 30 min. earlier than UV-induced organisms. These results are in agreement with other cases reported in the literature, when organisms induced by agents other than UV irradiation, showed a shorter latent period than UV-induced organisms (Jacob, 1954).

Effect of deoxyribose nucleic acid (DNA) on the inducing action of phagolessin A58

It was reported in an earlier publication (Asheshov *et al.* 1952) that the inactivation of free phage particles by phagolessin A58 was inhibited by DNA. DNA also inhibited the inducing action of phagolessin A58. The usual

procedure in these experiments was to add DNA to the solution of antibiotic to give a final DNA concentration of 0.01% (w/v). When such mixtures were added to lysogenic *Escherichia coli* FCB organisms, no induction occurred. When the DNA was added after induction had begun it prevented any further induction but was unable to affect cells which were already induced at the time of its addition.

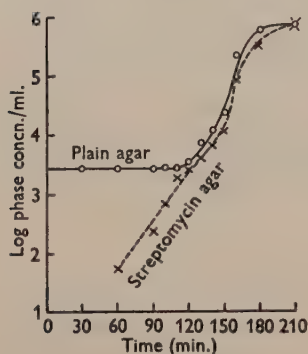


Fig. 9

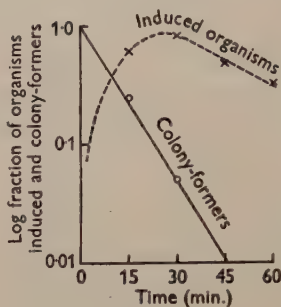


Fig. 10

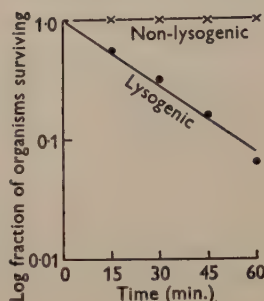


Fig. 11

Fig. 9. Effect of streptomycin on UV-induced *Escherichia coli* FCB. Organisms, induced by exposure to UV radiation for 15 sec. were diluted in nutrient broth and incubated at 37°. At regular intervals platings were made on plain and on streptomycin agar plates to count clearings. The log of the phage concentration/ml. is plotted against the time after irradiation. 0 hr. = time of dilution in nutrient broth.

Fig. 10. Induction of *Escherichia coli* FCB by phagolessin A58, expressed as a function of the time of exposure. The organisms were exposed to 15 units phagolessin A58/ml. in M/50 phosphate buffer at 37°. At regular intervals samples were removed, diluted in nutrient broth, and plated to count colony-formers and organisms induced. The log of the fraction of colony-formers and organisms induced is plotted against the time of exposure.

Fig. 11. A comparison of the sensitivity to phagolessin A58 of lysogenic *Bacillus megaterium* 899 and its non-lysogenic mutant. Organisms of each of the two strains, in the log phase of growth, were washed, suspended in M/50 phosphate buffer and exposed to 15 units phagolessin A58/ml. at 37°. At 15 min. intervals samples were removed, diluted in nutrient broth and plated to count colony-formers. The log of the fraction of organisms able to form colonies is plotted against time of exposure.

Effect of phagolessin A58 on the other three lysogenic cultures

Experiments similar to those described above were carried out with the other three lysogenic organisms. *Bacillus megaterium* 899 was induced by exposure to phagolessin A58 and results similar to those described with UV irradiation were obtained with the exception that the latent period was reduced to 45 min. (i.e. approximately 20 min. shorter than was the case with UV-induced *B. megaterium* 899). Induction was inhibited in the presence of DNA but was not reversed by adding DNA once it was established.

Exposure of *Escherichia coli* Temple or *E. coli* Lampert to phagolessin A58 did not cause any apparent induction. There was a loss in colony-formers

following such treatment but there was no increase in phage titre. We concluded, therefore, that these two lysogenic strains are not inducible by either UV irradiation or by phagolessin A58.

The use of phagolessin A58 to select non-lysogenic mutants

Phagolessin A58 can be considered to have two separate actions on lysogenic *Bacillus megaterium* 899 and *Escherichia coli* FCb, and their carried phages. First, the antibiotic induces the organisms to lyse and release their phage (the inducing action); secondly, the antibiotic inactivates the free particles released by the lysing organisms (the phagicial action). It seemed that, with these two properties, it should be possible to select non-lysogenic phage-sensitive mutants by growing the lysogenic organisms over a period of time in the presence of the antibiotic. The combined inducing and phagicial actions should provide conditions favourable to the selection of such mutants provided that they arise fairly frequently during the growth of lysogenic organisms. Phage 899 requires calcium ions for some stage in its intracellular development in sensitive organisms (Wahl, 1946). Clarke (1952) produced a non-lysogenic phage-sensitive mutant of *Bacillus megaterium* 899 after 61 subcultures of the lysogenic culture in calcium-deficient medium. Lwoff (1953) confirmed these results and isolated such a mutant after 25 to 34 cultures. The phagicial action of phagolessin A58 might be considered to correspond to the effect of calcium deficiency since in both cases non-lysogenic phage-sensitive mutants would be protected from the action of free phage particles. The additional inducing action of phagolessin A58 should further aid in the selection by eliminating lysogenic organisms—i.e. by causing them to lyse. One would expect, therefore, that transfers of *B. megaterium* 899 in the presence of phagolessin A58 would produce a non-lysogenic phage-sensitive mutant more rapidly than cultivation of the lysogenic organisms in calcium-free medium. In theory it should also be possible to obtain similar results with *Escherichia coli* FCb since both the inducing and phagicial actions would be operating. With the other two lysogenic strains (*E. coli* Temple and *E. coli* Lampert), which are not inducible, only the phagicial action should operate.

Accordingly, attempts were made to produce non-lysogenic phage-sensitive mutants of the four lysogenic strains by means of serial subculture in nutrient broth containing phagolessin A58. Two concentrations of phagolessin A58 were used—10 or 20 units/ml. Fairly large numbers of organisms were transferred each time to give a starting inoculum, at each transfer, of $c. 10^5$ organisms/ml., in order to increase the chance of picking up any mutants that might be present. Subcultures were made every 12 hr. and the tubes were incubated at 37°.

The subcultures were examined at intervals to see whether the treatment had affected the lysogenicity of the strain. With *Bacillus megaterium* 899 the method was quite simple. The 12 hr. growth of the subculture being tested was centrifuged down and the supernatant fluid assayed for free phage particles. The organisms were resuspended in nutrient broth, and suitable dilutions plated on plain agar plates and on plain agar plates first inoculated with the

indicator strain. The number of colonies which developed on plain agar gave the total cell concentration, while the number of colonies which were surrounded by the typical lytic zone when plated on the indicator strain gave the total number of lysogenic organisms. A difference between the total count of organisms and the lysogenic organism count indicated that non-lysogenic organisms were present. With this method it was possible to calculate the percentage of non-lysogenic mutants present in any subculture. As a further confirmation, individual colonies were isolated and tested for lysogenicity and sensitivity to phage 899. This method could not be used with the three lysogenic strains of *Escherichia coli* since it is not possible to distinguish between lysogenic and non-lysogenic organisms by plating on the appropriate indicator strain. However, by using Bertani's streptomycin technique (Bertani, 1951) it was a simple matter to assay the subcultures for free phage concentration. The 12 hr. growth was suitably diluted and plated on streptomycin agar plates with the streptomycin-resistant mutant of the indicator strain. The concentration of free phage particles in the treated tubes was compared with that in the untreated control. As an additional check on the lysogenicity of a particular subculture, the growth was plated out and individual colonies, isolated into nutrient broth, were tested for phage production after several hours of incubation at 37° by the streptomycin technique.

We were successful only with *Bacillus megaterium* 899. With this organism it was usually possible to observe the appearance of non-lysogenic mutants after 4 to 5 subcultures. In one experiment, 28% of the organisms were non-lysogenic in the 4th subculture, 96% in the 10th subculture, and the 12th subculture contained no free phage particles and appeared to consist entirely of non-lysogenic organisms. Not all such experiments with this strain were successful, however, and on two occasions subcultivation resulted in a complete sterilization after 4 to 6 subcultures. The non-lysogenic mutants that were isolated were all sensitive to phage 899, and were also highly resistant to phagolessin A58. When suspensions of the mutant in M/50 buffer were exposed to 15 units phagolessin A58/ml. for 1 hr. at 37° there was no decrease in the number of colony-formers. During the same period of time the original lysogenic strain showed a loss of some 93% of viable organisms as judged by their colony-forming ability (Fig. 11). The non-lysogenic mutant could be re-lysogenized by exposing it to phage 899, and the re-lysogenized culture showed the same sensitivity to phagolessin A58 as the original lysogenic strain. It is the presence of the prophage in the lysogenic organism, therefore, which is responsible for the sensitivity of the lysogenic organism to phagolessin A58.

Subcultivation of the three lysogenic strains of *Escherichia coli* in phagolessin A58 had no effect on the lysogenicity of the organisms. There was some inhibition in growth (particularly with *E. coli* FCb, which was to be expected in view of the inducing action of the antibiotic) but after 26 subcultures the organisms were still fully lysogenic. Moreover, the free phage concentration of the 12 hr. growth of the 26th subcultures was only slightly lower than in the untreated control. This failure with the three *E. coli* strains may be due to several factors. The emergence of a non-lysogenic mutant depends on the

frequency with which lysogenic organisms mutate to a non-lysogenic form, and it is possible that this mutation occurs less frequently in these three cultures than in *Bacillus megaterium* 899. Again, the sensitivity of phages FCb, Temple and Lampert to phagolessin A58 is much lower than that of phage 899, so that more free phage would survive to eliminate any non-lysogenic phage-sensitive mutants that might be present. Also, phagolessin A58 is very unstable in nutrient broth at pH 7.0 at 37°, about 50 % of its phagicial action is destroyed after *c.* 6 hr. in nutrient broth under these conditions. These facts probably explain the relatively high concentration of free phage in the treated tubes. It is possible that if subcultivation were made at shorter intervals we might have succeeded with these cultures. Attempts to grow the strains at antibiotic concentrations higher than 20 units/ml. were tried but were not successful since the cultures were sterilized.

DISCUSSION

Since the original discovery by Lwoff *et al.* (1950) that in certain lysogenic strains of bacteria the lytic cycle can be induced by UV irradiation, a number of other inducing agents have been discovered (Lwoff & Simonovitch, 1952; Lwoff & Jacob, 1952; Jacob, 1952*a*; Williams Smith, 1953). In general these inducing agents are also mutagenic or carcinogenic agents. Induction by these other agents resembles that by UV radiation in all except two respects. Organisms induced by UV radiation can be restored to their normal lysogenic state by illumination with visible light (photorestitution), provided that they are illuminated within 20–30 min. after induction (Jacob, 1950). Attempts to restore organisms induced by the other inducing agents by means of visible light have failed. Also, the length of the latent period of UV-induced organisms is 20–30 min. longer than that of similar organisms induced by any of the other agents. Jacob (1954) suggested that these two observations are related, and according to his hypothesis, the prophage in UV-induced organisms requires approximately 20–30 min. to pass into the vegetative state, and until the vegetative state has been reached, such organisms are restorable. Similar organisms, induced by agents other than UV radiation, pass immediately into the vegetative state and are, therefore, not restorable, and have a latent period which is shorter than that of UV-induced organisms by *c.* 20–30 min.

Phagolessin A58 resembles these other inducing agents rather than UV radiation with regard to the restorability of the induced organisms and the length of the latent period. Apart from these two differences there is a parallelism between induction by UV radiation and by phagolessin A58 in that strains which are inducible by one agent are also inducible by the other. Inducibility therefore seems to be a property of the particular lysogenic strain and can be initiated by a variety of agents. Ionesco (1951) and Jacob (1952*b*) showed that inducibility is a property of the prophage or the prophage-host cell complex and not of the particular bacterial strain which carries the prophage.

A similar parallelism was noted between the action of UV radiation and phagolessin A58 on free phage particles (Hall & Asheshov, 1953). Treatment

of free phage particles with phagolessin A58 resulted in a loss of infectivity, but the inactivated particles remained capable of being adsorbing to and killing sensitive host organisms. These results with free phage particles suggest that the action of phagolessin A58 is directed against the nucleic acid of the particle rather than the protein fraction, since it has been shown (Herriott, 1951) that it is the protein fraction which is responsible for adsorption to and killing of sensitive host organisms. Further support to this theory is lent by the fact that DNA specifically inhibits the action of phagolessin A58 on free phage particles. DNA also inhibits the inducing action of phagolessin A58 and it is tempting to speculate that, in this instance, the site of action is the DNA of the lysogenic cell.

Weigle & Delbrück (1951) suggested that the inducibility of a lysogenic strain might depend on the susceptibility of the carried phage to the inducing agent. With *Escherichia coli* strain K-12 they showed that this organism, which is inducible by UV radiation, carries a phage which is unusually resistant to UV radiation; Jacob (1954) reported similar results with lysogenic strains of *Pseudomonas aeruginosa* (*pyocyanea*). Induction of lysogenic strains by phagolessin A58 is independent of the sensitivity of the carried phage to the antibiotic. Of the phages carried by the four different lysogenic strains used in the present work, phage 899 is the most sensitive and phage FCb the least sensitive to inactivation, yet both of these lysogenic strains are inducible by phagolessin A58. On the other hand, phage Temple and phage Lampert, lying midway between the other two phages with regard to their sensitivity to phagolessin A58, are carried by two non-inducible strains. Jacob (1952*b*) and Lwoff (1953) have both pointed out that inducible lysogenic strains are those strains which have a higher probability of undergoing spontaneous lysis. Our results are in agreement with this, since both *Bacillus megatherium* 899 and *Escherichia coli* FCb show a greater probability of lysing spontaneously than either of the other two strains, judging by the amount of free phage present in actively growing broth cultures of the four strains.

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The Occurrence of Chitinase in some Bacteria

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SUMMARY: A method for the detection of small quantities of chitinase in culture fluids is described and the results of a survey of representative species of the main groups of bacteria presented. Chitinase is produced by some but not all of the soil and water bacteria. Chitinase appears to be a constitutive enzyme in many species of bacteria; its occurrence may prove of diagnostic value.

Chitin decomposition is known to be initiated by a large number of bacterial species isolated from marine deposits or from soil (Campbell & Williams, 1951; Veldkamp, 1955). During a search for a convenient source of chitinase (Tracey, 1955*b*) a number of cultures of bacteria of medical provenance as well as soil and plant pathogenic organisms were examined. A surprising number of organisms, including human pathogens, was found to produce chitinase, and it seems possible that this property may be of diagnostic value.

METHODS

Organisms were grown at Rothamsted on nutrient broth (10 g. peptone; 3 g. Lemco; 1 g. Marmite; 5 g. NaCl; made to 1 l. with distilled water; pH 7·2) or on nutrient agar. Organisms grown by P. H. C. working at the National Collection of Type Cultures (NCTC) were grown in nutrient broth (10 g. peptone; 10 g. Lemco; 5 g. NaCl; water to 1 l; pH 7·4) with the exception of the clostridia which were grown in meat broth containing particles of meat. Cultures were incubated with toluene overnight before use.

Chitin. The shells of *Sepia officinalis* were used as a source (they are commercially available as cuttlefish 'bones'; imported usually from Portugal or India where they are gathered in a dry bleached condition from the beaches). After soaking in dilute HCl each shell disintegrates into one thick chitinous layer (the pro-ostacum) and a large number of chitinous sheets (about 7 μ . thick) with irregular surfaces. The yield from the shells is about 3 %, and about two-thirds of the dry weight of the sheets is chitin. This material was dispersed in cold conc. HCl and, after centrifugation or filtration through glass wool, poured into 10 vol. of water, with vigorous stirring. The precipitated finely divided chitin was washed and concentrated by decantation and a thick suspension finally dialysed against running distilled water. A suspension containing 100 μ g. chitin N/ml. was used as the stock substrate and preserved with toluene.

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Chitinase. After incubation overnight with toluene cultures were centrifuged and 2 ml. of the supernatant mixed with 2 ml. of pH 5 acetate buffer (0.2M) and 1 ml. of chitin suspension in centrifuge tubes. Controls of the same total volume containing culture fluid without chitin, and with chitin and buffer alone were also prepared. To all tubes a few drops of toluene were added, the tubes stoppered and the contents mixed. After incubation at 37° for 1, 3 and 8 days the tubes were centrifuged and the acetylglucosamine content of the supernatant fluid determined. When the organisms produced indole a determination before incubation with chitinase was made. The presence of acetylglucosamine was detected by a modification of the Morgan & Elson (1934) method (Tracey, 1955*a*). In this method a colour is formed by the reaction of *p*-dimethylaminobenzaldehyde (Ehrlich reagent) with the product formed by heating acetylglucosamine in alkaline solution. A colour is also produced in this reaction by acetylgalactosamine and by blood group mucoids (Aminoff, Morgan & Watkins, 1952) but not by the disaccharide (*N, N'*-diacetylchitobiose) which is the simplest product, other than acetylglucosamine, of the incomplete enzymic hydrolysis of chitin (Reynolds, 1954; Kuhn, Gauhe & Baer, 1954). Under the conditions used indole gives a very similar colour (indole colour absorbs maximally at 565 m μ .; acetylglucosamine colour at 580 m μ .) with about 2.5 times the intensity of that given by acetylglucosamine of equimolar concentration. Thus the appearance of a pink colour after the reaction has been carried out indicates the presence of free acetylglucosamine, indole or possibly breakdown products of chitin other than *N, N'*-diacetylchitobiose. In centrifuged fluids from toluene-killed cultures there was no further production of indole on incubation while in the presence of chitinase the production of material which reacted with Ehrlich reagent after alkaline heating was continued for many days. Only in the presence of indole was any colour produced in the controls. Consequently, chitinase may be detected in culture fluids from indole-producing organisms since in its presence there is a steady increase in the colour produced in the presence of chitin but no increase in its absence. It seems probable that the material responsible for increased colour production as a result of incubation with chitin is acetylglucosamine alone, though it is possible that large fragments resulting from the breakdown of chitin may react in a manner similar to the blood group mucoids. Should this be so, colour production is still indicative of chitin breakdown and hence of the presence of chitinase, for this term may apply to a number of enzymes of different properties with different end products of hydrolysis.

In the absence of indole the presence of 0.25 μ g. acetylglucosamine-*N* can be detected in 1 ml. of fluid after incubation with chitin corresponding to the breakdown of a total of 18 μ g. chitin (1.25 μ g. chitin-*N*). In the presence of indole a rather greater breakdown must have occurred for a positive result to be recognized.

Cellulase. Some cellulase determinations were made on a few culture fluids by a viscometric technique, with sodium carboxymethylcellulose as substrate (Tracey, 1950).

Indole production. Indole production was also detected by the use of oxalic acid papers inserted above cultures growing in Lemco broth at 37°.

Methyl-red test. Carried out by growing cultures in glucose phosphate peptone medium and incubating for 5 days at 30° (%, w/v: glucose 0.5; peptone 0.5; K₂HPO₄ 0.5) and by the methods described by Jennens (1954).

Voges-Proskauer test. Carried out on three media: glucose + phosphate + peptone medium; glucose + peptone medium with NaCl in place of phosphate (Smith, Gordon & Clark, 1946); and glucose + peptone medium without salt or phosphate (Abd-el-Malek & Gibson, 1948). Cultures were incubated at 30° for 5 days and tested for the presence of acetoin by Batty-Smith's (1941) modification of Barritt's (1936) α -naphthol method.

RESULTS

Liberation of enzyme

About 100 mg. wet weight of *Klebsiella* (*Aerobacter*) sp. grown on nutrient agar were suspended in water. Three treatments were compared for enzyme liberation. Incubation overnight with 3M-glycine (Maculla & Cowles, 1948) resulted in the liberation of 1.2 units of cellulase, freezing and thawing four times resulted in the presence of 25 units in the supernatant fluid and incubation with toluene overnight at 37° of 49 units.

Effect of conditions of growth

Six irregular coliform organisms were grown on nutrient broth for 2, 4 and 6 days at 37°. Maximum chitinase activity was reached at 2 days for one strain, at 4 days for a second and at 6 days for the remainder. These cultures were all of low chitinase activity. An active organism, *Pseudomonas hydrophila* (NCTC 7810), was grown in nutrient broth and in similar broth containing 1% glucose. Chitinase production was decreased by a factor of 3-5 by the addition of glucose. Five to six days of incubation at 37° without added glucose was adopted as a routine for the growth of cultures.

Table 1. *Effect of varied conditions of growth on the production of cellulase and chitinase, by two Klebsiella species*

Toluene-killed cultures incubated 3 days with appropriate substrates; carboxymethyl-cellulose for cellulase, finely divided chitin for chitinase.

	Medium					
	Nutrient agar plates		Sloppy agar		Broth	
	Cellulase	Chitinase	Cellulase	Chitinase	Cellulase	Chitinase
<i>K. pneumoniae</i>						
NCTC 5054	0.175	0.021	< 0.025	0.022	< 0.025	0.022
NCTC 5055	0.110	0.019	< 0.025	0.010	< 0.025	0.013
<i>K. rhinoscleromatis</i>						
NCTC 5046	0.195	0.013	< 0.025	0.010	< 0.025	0.017
NCTC 7799	0.170	0.016	< 0.025	0.016	< 0.025	0.014

Figures are for total reducing sugar and total acetylglucosamine formed respectively.

A comparison of enzyme production on liquid and solid media showed that the ratio of cellulase activity to chitinase activity in *Klebsiella pneumoniae* and *K. rhinoscleromatis* was markedly affected by change of medium. Accordingly, a comparison was made between organisms grown on nutrient broth, on similar broth containing 0.05 % agar (insufficient to solidify it) and on broth containing 1.5 % agar poured into Petri dishes. It is apparent from the results (Table 1) that the detection of cellulase production is easier when the organisms are grown on the solid medium, while the ease of detection of chitinase is unaffected. The presence of agar in the liquid medium had no effect on the relative amounts of the enzymes.

No chitin was added to the media used for the growth of the organisms and none was detected in it by chemical analysis. Incubation with a powerful chitinase from *Lycopodon pyriforme* (Tracey, 1955a) did not result in the formation of any detectable acetylglucosamine. If any chitin was present its concentration must have been < 6 mg./l. No acetylglucosamine was detected in the medium.

Properties of the enzyme

That the enzyme (or enzymes) concerned in the breakdown of chitin is fairly stable is indicated first by its withstanding incubation with toluene overnight in the absence of substrate and secondly by the fact that acetylglucosamine production from chitin at 37° normally continues at a fairly steady rate for 10 days or more. *Pseudomonas hydrophila* extracts were considerably more active at pH 5 than at pH 7. Some of the organisms were tested for the ability to de-acetylate the acetylglucosamine produced. With no organism tested was there any evidence of the production of glucosamine even after prolonged incubation. Since the cultures used were killed it is unlikely that de-acetylation occurred immediately followed by destruction of any glucosamine formed.

It is known that some cellulase preparations are more active in the breakdown of cellulose in the presence of added protein (Whitaker, 1952; Tracey, 1953). This effect has also been found for some fungal chitinases (Tracey, 1955b). The effect of adding protein to the chitinase assay medium was therefore tried. The addition of a drop of dialysed bovine serum to the contents of the tubes in which chitin was incubated with the extracts materially increased the activity observed. The amount of acetylglucosamine produced during a given interval was increased in the presence of the added protein by a factor varying from 2 to 5. The variation was probably related to the variation in protein concentration of the extracts tested. This activation by protein enables definite results to be obtained more quickly with weak chitinase producers and was later adopted as a routine, dialysed bovine serum also being added to the control tubes.

Distribution of chitinase

A survey of representative species of the main groups of bacteria showed that chitinase was produced by some but not all of the soil and water organisms (Table 2). The most active preparations were obtained from species of

Table. 2. *Distribution of chitinase among various bacteria*

Organism	Reference number	Chitinase production
<i>Chromobacterium essayanum</i>	NCTC 4618	+
<i>C. indicum</i>	2847*	+
<i>C. prodigiosum</i>	3804	+
<i>C. prodigiosum</i>	4612	+
<i>C. violaceum</i>	8683	+
<i>Klebsiella aerogenes</i>	RES 07	—
<i>K. aerogenes</i>	RES 1912*	+
<i>K. aerogenes</i>	NCTC 8167	—
<i>K. aerogenes</i>	418	+
<i>Pseudomonas hydrophila</i>	7810*	+
<i>P. ichthyosmia</i>	8049*	+
<i>P. pyocyanea (aeruginosa)</i>	RES 2650	+
<i>Pseudomonas</i> sp.	RES 4002	—
<i>Pseudomonas</i> sp.	RES 4036	—
<i>Clostridium oedematiens</i> type A	NCTC 277	—
<i>C. septicum</i>	284*	+
<i>C. septicum</i>	285*	+
<i>C. sporogenes</i>	534	—
<i>C. tetani</i>	5410	—
<i>C. welchii</i> type A	8246*	+
<i>C. welchii</i> (heat resistant)	8449	—
<i>Vibrio cholerae</i> subgroup I	6560*	+
<i>V. cholerae</i> subgroup I	7252*	+
<i>V. cholerae</i> subgroup I	8022	—
<i>V. el Tor</i>	4714*	+
<i>Vibrio</i> sp.	30	—
<i>Vibrio</i> sp.	4716*	+
<i>Vibrio</i> sp.	8042	—
<i>Escherichia coli</i>	8164	—
<i>E. coli</i>	8169	—
<i>E. coli</i>	8170	—
<i>E. coli</i>	8196	—
<i>E. coli</i>	RES	—
<i>Erwinia aroideae</i>	M 16	—
<i>E. aroideae</i>	RES	—
<i>E. carotovora</i>	M 14	—
<i>Erwinia</i> sp.	RES	—
<i>Erwinia</i> sp.	M 13	—
<i>Erwinia</i> sp.	M 23	+
<i>Proteus mirabilis</i>	NCTC 3156	—
<i>P. mirabilis</i>	6789	—
<i>P.morganii</i>	1709	—
<i>P.morganii</i>	2814	—
<i>P. rettgeri</i>	7477	—
<i>P. rettgeri</i>	7478	—
<i>Salmonella enteritidis</i>	NCTC 3046	—
<i>S. paratyphi-A</i>	8012	—
<i>S. paratyphi-B</i>	8458	—
<i>S. paratyphi-C</i>	8053	—
<i>S. typhi</i>	779	—
<i>S. typhimurium</i>	3173	—
<i>S. typhisuis</i>	5739	—
<i>Shigella boydii</i>	8214	—
<i>S. dysenteriae</i>	6338	—
<i>S. dysenteriae</i>	8005	—
<i>S. dysenteriae</i>	8019	—

Table 2 (cont.)

Organism	Reference number	Chitinase production
<i>S. dysenteriae</i>	8379	—
<i>S. flexneri</i>	2	—
<i>S. flexneri</i>	7885	—
<i>S. flexneri</i>	8061	—
<i>S. flexneri</i>	8192	—
<i>S. sonnei</i>	7240	—
<i>Bacillus alvei</i>	7583	—
<i>B. laterosporus</i>	7579	—
<i>B. macerans</i>	7588	—
<i>B. sphaericus</i>	7582	—
<i>Corynebacterium viscosum</i>	2416	—
<i>Mycobacterium smegmatis</i>	525	—
<i>M. phlei</i>	RES	—
<i>Nocardia lutea</i>	RES	—
<i>Staphylococcus aureus</i>	8349	—
<i>S. aureus</i>	8353	—
<i>S. aureus</i>	8355	—
<i>S. aureus</i>	8357	—
<i>S. aureus</i>	8363	—
<i>Staphylococcus</i> sp.	952	—
<i>S. sp. (Sarcina flava)</i>	RES 611	—
<i>S. sp. (Micrococcus sp.)</i>	RES T20	—
<i>Streptococcus pyogenes</i>	8328	—
<i>Pasteurella pseudotuberculosis</i>	1101	—
<i>P. pseudotuberculosis</i>	2478	—
<i>P. septica</i>	1287	—
<i>P. septica</i>	4881	—
<i>Rhizobium trifolium</i>	RES Coryn	—
<i>R. trifolium</i>	RES C1F	—
<i>R. trifolium</i>	RES A11	—
<i>R. trifolium</i>	RES HKC	—
<i>R. trifolium</i>	RES A121111	—

NCTC numbers unless marked otherwise; *=reaction enhanced by added protein; absence of mark from positive organisms means protein effect not tested. RES=cultures from Rothamsted Experimental Station; M=cultures supplied by Dr J. F. Malcolmson.

Chromobacterium, *Pseudomonas* and *Klebsiella*. Two *Pseudomonas* spp. isolated from soil failed to produce chitinase, and *P. hydrophila* NCTC 7810 appeared to have lost the ability to produce chitinase when retested 2 years later (other organisms retested had retained full activity). Weaker reactions were given by some species of water vibrios and clostridia. *Rhizobium trifolium* strains were all negative; one *Erwinia* culture produced chitinase.

Of particular interest were the reactions of the enterobacteria. *Klebsiella aerogenes* strains produced chitinase, whereas *Escherichia coli* in common with the salmonellas was negative. Table 3 shows that chitinase production among the coliform organisms was mainly confined to the *Klebsiella* group. Of the *Klebsiella aerogenes* I (indole-negative) cultures five were chitinase-positive and one chitinase-negative. Of the *K. aerogenes* II (indole-positive) cultures, five were chitinase-negative and one (NCTC 8848) doubtful. All the strains tested of *K. cloacae*, *K. ozaenae*, *K. pneumoniae* and *K. rhinoscleromatis* were chitinase producers. Six cultures tested which had been classified as coliform irregular

were tested; all except NCTC 8866 produced indole and gave positive methyl red and Voges-Proskauer reactions when tested on peptone media. Jennens (1954) showed that in the inorganic medium of Fouad & Richards (1952) they were methyl-red negative. These six cultures all gave a weak chitinase reaction.

Table 3. *Chitinase production among coliform bacteria*

Species	No.	Chitinase	Indole	V.P.	MR.
<i>Klebsiella aerogenes</i> I	418	+	—	+	—
<i>K. aerogenes</i> I	243	+	—	+	—
<i>K. aerogenes</i> I	8197	+	—	+	—
<i>K. aerogenes</i> I	M 4	—	—	n.t.	n.t.
<i>K. aerogenes</i> I	M 20	+	—	n.t.	n.t.
<i>K. aerogenes</i> I	RES 1912*	+	—	n.t.	n.t.
<i>K. aerogenes</i> II	8167	—	+	+	—
<i>K. aerogenes</i> II	8801	—	+	+	—
<i>K. aerogenes</i> II	8824	—	+	+	—
<i>K. aerogenes</i> II	8848	+?	+	+	—
<i>K. aerogenes</i> II	8874	—	+	+	—
<i>K. aerogenes</i> II	RES 07	—	+	n.t.	n.t.
<i>K. cloacae</i>	5920	+	—	+	—
<i>K. cloacae</i>	6027	+	—	+	—
<i>K. cloacae</i>	8155	+	—	+	—
<i>K. cloacae</i>	8168	+	—	+	—
<i>K. ozaenae</i>	5051	+	—	+	—
<i>K. ozaenae</i>	5053	+	—	+	—
<i>K. pneumoniae</i>	5054	+	—	+	—
<i>K. pneumoniae</i>	5055	+	—	+	—
<i>K. rhinoscleromatis</i>	5046	+	—	+	—
<i>K. rhinoscleromatis</i>	7799	+	—	+	—
<i>Escherichia coli</i> I	8169	—	+	—	+
<i>E. coli</i> I	8196	—	+	—	+
<i>E. coli</i> II	8164	—	—	—	+
<i>E. coli</i> II	8170	—	—	—	+
<i>E. coli</i> II	RES	—	—	n.t.	n.t.
<i>E. coli mutabile</i>	8450	—	+	—	+
<i>E. intermedium</i>	6071	—	+	—	+
<i>E. intermedium</i>	8165	—	—	—	+
<i>E. intermedium</i>	8166	—	—	—	+
Irregular coliforms	8835	+†	+	+	+—°
Irregular coliforms	8844	+†	+	+	+—°
Irregular coliforms	8845	+†	+	+	+—°
Irregular coliforms	8847	+†	+	+	+—°
Irregular coliforms	8851	+†	+	+	+—°
Irregular coliforms	8866	+†	—	+	+—

NCTC numbers unless marked otherwise; n.t.=not tested; †=reactions weak; °+ in peptone media, — in glucose salt medium; *=reaction enhanced by added protein, absence of mark from positive organisms means protein effect not tested. RES=cultures from Rothamsted Experimental Station; M=cultures from Dr J. F. Malcolmson.

Distribution of cellulase

All strains tested of *Klebsiella pneumoniae*, *K. ozaenae* and *K. rhinoscleromatis* produced cellulase, as did the only strain of *K. aerogenes* tested (RES 1912). Strains of *Escherichia freundii* and *E. coli mutabile* were negative. Five strains of *Rhizobium trifolium* and single cultures of *Erwinia aroideae*,

Nocardia lutea, *Mycobacterium smegmatis* and *M. phlei* were negative. Among this small group cellulase was present whenever chitinase was found and absent in the organisms that were chitinase negative.

DISCUSSION

The method described allows the detection of an amount of enzyme breaking down 20 μ g. of precipitated chitin after 10 days at 37°. It may therefore be more sensitive than methods which depend on halo production by colonies grown on agar containing suspended chitin. When the result is positive there seems at present to be little likelihood of its being false. However, it is possible that false negatives may be found. In the present work no evidence of de-acetylation of acetylglucosamine during incubation was obtained with any of the cultures tested. The effect of de-acetylation is to produce a false negative, for glucosamine gives no colour with the method used. Should deacetylation occur it may be detected by testing the supernatant fluids for glucosamine as well as acetylglucosamine. This method (which involves heating the solution with an alkaline solution of acetylacetone followed by colour development under rather different conditions with Ehrlich reagent) has the disadvantage that both acetylglucosamine and glucosamine give a positive reaction. On an equimolar basis, however, colour production by glucosamine is six to seven times as great as with acetylglucosamine. Roseman (1954) reported the presence in cell-free extracts of *Escherichia coli* of an enzyme which de-acetylated acetylglucosamine; the optimum pH value was 8.3, no activity was detectable in pyrophosphate buffer and activity was enhanced when acetylglucosamine was present in the growth medium. It is possible that the absence of acetylglucosamine from the growth medium and incubation at pH 5 would have rendered de-acetylation inappreciable in the experiments reported in the present paper.

The absence (so far as could be determined) of chitin, of glucosamine or acetylglucosamine polymers and of acetylglucosamine from the growth medium would suggest that chitinase is a 'constitutive' enzyme whose presence depends neither on the presence of the substrate nor of its products of hydrolysis in the medium. A similar suggestion for the nature of some bacterial cellulases was advanced by Hammerstrom, Claus, Coghlan & McBee (1955).

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The Classification and Nomenclature of Organisms of the Pleuropneumonia Group

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The taxonomic status of the micro-organisms of the pleuropneumonia group has remained confused for many years. There is no generally accepted nomenclature or classification, although the need for these has been widely recognized and has become more urgent with the increasing number of new strains of pleuropneumonia-like organisms isolated. Recently Freundt (1955) and Edward (1955) made proposals for classifying and naming these organisms. Although published separately their proposals were in close agreement with each other, as a result of previous informal exchanges of views between the authors. However, it was felt that the publication of two separate papers, each of which provided lists of named species differing from each other only slightly, might cause confusion, particularly as both papers unfortunately contained a number of printing errors. Therefore, it was decided to prepare a joint paper. It is hoped that the proposals made will provide a basis for discussion among those interested in this group of organisms, and will lead to the adoption of a nomenclature which commands general agreement. It is also felt that the study of strains of pleuropneumonia-like organisms may be stimulated by the establishment of named species.

GENERAL RELATIONSHIPS

Relation to bacteria

The relation of the pleuropneumonia-like organisms to bacteria (Schizomycetes) requires special consideration because of its fundamental taxonomic importance. In this connexion particular attention must be paid to morphology and mode of reproduction. Unfortunately there is still considerable disagreement among workers who have studied these properties. Some authors (Ørskov, 1927, 1942*b*; Freundt, 1952*a*, 1954) have described a mycelial mode of growth occurring under optimal culture conditions with multiplication by the formation in the filaments of minute endomycelial corpuscles which are subsequently released as free elementary bodies. This method of reproduction has certain features in common with that of some *Actinomyces* spp. The pleuropneumonia-like organisms also resemble the actinomycetes in the way their colonies grow into the media. If this interpretation of morphology and method of reproduction, which dates back to the earliest investigations of the organisms (for references see Freundt, 1952*a*), were generally accepted it would warrant

the classification of the pleuropneumonia group of organisms as a true bacterial order, parallel with, but not actually phylogenetically related to, Actinomycetales.

Reproduction by other methods has, however, been described. Keller & Morton (1954) and Butler & Knight (1956), using turbidimetric and viable count methods, demonstrated a normal bacterial growth cycle with strains of pleuropneumonia-like organisms, thus suggesting growth by simple binary fission. A quite different method of multiplication has been claimed by Klieneberger-Nobel (1954) and Dienes & Weinberger (1951), who believed that small granules multiply within the cytoplasm of large, round cells; after rupture of the surrounding membrane these granules develop into large cells and repeat the life cycle. The L-phase variants of bacteria were believed to multiply in a similar manner (Dienes & Weinberger, 1951; Tulasne, 1951). Reproduction by budding, similarly to yeasts, has also been claimed for pleuropneumonia-like organisms (Turner, 1935).

The cytoplasm of true bacteria is enclosed within a definite, more or less rigid, cell wall, but the elementary bodies and filaments of pleuropneumonia-like organisms are bounded only by a delicate membrane; the organisms are thus extremely plastic and easily ruptured or distorted by mechanical influences, such as changes in osmotic pressure. Although this cytological difference between pleuropneumonia-like organisms and bacteria is obviously important, further studies, particularly in regard to the physiological functions which are linked with the cell surface, are required before its significance can be fully evaluated.

The study of the metabolic behaviour of the pleuropneumonia-like organisms is still in its infancy, although remarkable progress has been made in recent years and a number of differences shown between pleuropneumonia-like organisms and bacteria. Pleuropneumonia-like organisms exhibit a greater resistance to a number of bacteriostatic substances. For instance, they will resist concentrations of crystal violet which inhibit Gram-positive bacteria and will also grow in concentrations of potassium tellurite which are inhibitory for Gram-negative bacteria (Edward, 1947; Smith, Morton & Leberman, 1950). Probably of greater importance is the absolute resistance to penicillin, independent of the action of penicillinase. Cholesterol, or certain other sterols, is an essential nutrient for pleuropneumonia-like organisms, with the possible exception of the saprophytic strains (Edward & Fitzgerald, 1951*b*). Media that have not been enriched with serum permit the growth of the saprophytic strains, but are likely to contain small amounts of sterol. The possibility of growing the strains in strictly sterol-free media has not been investigated. The finding that cholesterol is a growth factor would seem to be particularly significant, for sterols have not been found to be essential for the growth of bacteria, even in their L-phase. The value of a difference in an essential growth factor in distinguishing between large groups of micro-organisms has been recognized previously; Knight (1955) noted that *m*-inositol is required as a nutrient by many eumycetes, whereas it has never been found to be required as a nutrient by any schizomycete.

The growth of pleuropneumonia-like organisms can be inhibited by incorporation of an antiserum in the culture medium; complement is not concerned in this inhibition. Although growth of *Leptospira* spp. is inhibited by antisera, no other bacterium is known to be inhibited in a similar way. Analogies have been drawn between this type of inhibition and neutralization of viruses by antibody (Edward & Fitzgerald, 1954).

To conclude, obvious differences exist between pleuropneumonia-like organisms and bacteria. Sabin (1941*a*), Edward (1954) and Klieneberger-Nobel (1954) in their reviews of the organisms of the pleuropneumonia group regarded these differences as being sufficiently great to warrant classifying them separately from both bacteria and viruses. However, the taxonomic significance of these differences is still largely a matter of opinion, and every worker may not agree that the evidence is sufficient to exclude the pleuropneumonia-like organisms from the Schizomycetes. Final decisions must await further investigation.

Relation to the L-phase variants of bacteria

The disagreement regarding the relationship of organisms of the pleuropneumonia group to the L-phase variants of bacteria has for many years been one of the major obstacles to any attempt to define and classify the group. Dienes & Weinberger (1951) and Tulasne (1951) suggested that the pleuropneumonia-like organisms may have been derived from different bacteria through the formation of persistent L-phase variants. Such a view obviously excludes the possibility of classifying the organisms in a phylogenetically homogeneous group.

The similarities between organisms of the pleuropneumonia group and L-phase variants that have been advanced by those who adhere to this theory may be summarized as follows: an almost identical appearance of the colonies on solid media, morphological and physical similarity of the individual elements that make up the cultures, reproduction by swelling into large bodies and disintegration of these into granules, dependence on serum or ascitic fluid for growth and lack of sensitivity to penicillin.

In the experience of Ørskov (1942*a*), Klieneberger-Nobel (1954) and the present authors (Freundt, 1950; Edward, 1954), it is possible to distinguish L-phase colonies from those of organisms of the pleuropneumonia group, even on their gross appearance. A concrete example may illustrate this claim. Cultivation made by one of us (E. A. F.) from a submaxillary abscess of a rat yielded a mixed culture on solid medium comprising, amongst others, colonies of *Streptobacillus moniliformis*, together with two types of minute colony, one of which was at once regarded as the L-phase of *S. moniliformis* (L_1), while the other was suspected to be a true pleuropneumonia-like organism. Further study of pure cultures proved this original conception to be correct. The ' L_1 ' colonies reverted to streptobacilli, while the other type was identified as *Mycoplasma arthritidis* (L_4 of Klieneberger).

This is not the place to describe the detailed morphology of the individual elements of the two groups of organisms or to discuss the interpretation of these

features. It will be sufficient to draw attention to a few points only. The formation of elementary bodies in filamentous structures, as described above, which has been demonstrated by many workers with pleuropneumonia-like organisms, most recently by White, Wallace & Alberts (1954) in electron micrographs of avian strains, has never been reported in L-phase cultures, although small filaments are eventually found in these. The significance of the large bodies in any comparison between the two groups of organisms depends on the interpretation reached regarding their true nature, and on this there is still fundamental and profound disagreement between various workers. It may be mentioned, however, that attempts to elucidate the nature of the large bodies by various experimental methods have failed to support the conception that the minute granules formed within them are viable elements; this was investigated for the L-phase variants by Freundt (1950) and Kellenberger, Liebermeister & Bonifas (to be published) and for the pleuropneumonia-like organisms by Freundt (1952*b*), Keller & Morton (1954) and Kellenberger *et al.* If the large bodies are largely degenerative in nature, as suggested by Ørskov (1927, 1942*a, b*) and Freundt (1950, 1952*b*), it is obvious that claims for a relationship between the pleuropneumonia-like organisms and L-phase variants of bacteria cannot be based upon them.

There appear to be important differences between organisms of the pleuropneumonia group and the L-phase of bacteria in metabolism and growth-requirements. Both pleuropneumonia-like organisms (except the saprophytic species) and the L-phase of Gram-negative bacteria required media enriched with a substance such as serum. For the former group of organisms the serum appears to act by supplying cholesterol which is an essential nutrient. Cholesterol, however, was not essential for the growth of two L-phase organisms (Edward, 1953). The L-phase of *Proteus mirabilis* has in fact been cultivated in a defined medium containing a mixture of amino acids (Medill & O'Kane, 1954). The serum appears to promote the growth of this organism by neutralizing an inhibitor. It has been pointed out elsewhere that if pleuropneumonia-like organisms had been derived from different bacteria it would be expected that their fermentative capacity for various carbohydrates would vary from organism to organism, whereas it has been found that all pleuropneumonia-like organisms studied, if they ferment carbohydrates at all, ferment the same carbohydrates (Edward, 1954).

Recently Tulasne, one of the advocates of a close relationship between organisms of the pleuropneumonia group and L-phase variants, has in conjunction with Brisou (Tulasne & Brisou, 1955) suggested a classification and nomenclature in which pleuropneumonia-like organisms without a proven derivation from bacteria are placed in one order, Pleuropneumoniales, and the L-phase variants of bacteria are assigned to a second order, Bactepneumoniales. Thus these workers agree to grouping organisms of the pleuropneumonia group proper, separately from the L-phase organisms. Although the nomenclature of the L-phase organisms will not be considered here the comment might be made that grouping together the L-phase variants of diverse bacteria into a completely new order would seem open to grave objection.

Relation to viruses

Although the organisms of bovine pleuropneumonia and of agalactia were for many years regarded as viruses on account of their small dimensions, the pleuropneumonia-like organisms as a group are now generally regarded as distinct from the viruses. However, certain analogies with the viruses, although admittedly vague and merely suggestive, deserve mention.

Attention has been drawn previously (Edward, 1954) to an apparent resemblance in mode of reproduction, whereby filaments break up into elementary bodies, between pleuropneumonia-like organisms and influenza virus on the surface of infected cells (Wyckoff, 1953; Chu, Dawson & Elford, 1949). Nevertheless, it would probably be unwise at present to attach too much importance to this similarity, because it is still obscure how this phenomenon is to be properly interpreted for the influenza virus. Present evidence suggests that the homogeneous and fragmented filaments observed on cells infected with influenza virus develop from, and are continuous with, the cytoplasm of the cells, and may be a cellular response to infection.

Sabin (1941*a, b*) emphasized the specificity of species of pleuropneumonia-like organisms for particular hosts and used this as a basis for classification. This specificity appears to depend upon a very close relationship of the organisms when growing *in vivo* with certain host cells. Though perhaps not so definite and universal as suggested by Sabin, there is undoubtedly a tendency for specialized parasitic adaptation to the animal hosts which may be regarded as a distinctive feature, placing the pleuropneumonia group in this respect nearer to viruses than to most bacteria.

It was suggested by Lecce & Morton (1954) that the characteristically slow growth of the organisms of the pleuropneumonia group and their relatively limited metabolic activity may from an evolutionary standpoint be regarded as a step in the direction of increased nutritional dependency. The validity of this view seems somewhat doubtful because recent investigations with improved methods suggest that the metabolic activity of the organisms is not so restricted as was hitherto imagined.

TAXONOMIC UNITS

Type species

It can hardly be doubted that the organism of bovine pleuropneumonia (basonym: *Asterococcus mycoides*) is to be preferred as the type species. It was the first to be isolated, has been studied the most and has already provided the name by which the organisms of the group have hitherto been known (pleuropneumonia-like organisms). The choice by Sabin (1941*b*) of the organism of agalactia as the type species would have no advantage, and was in fact unfortunate, because its cultural properties had not then been firmly established (Edward, 1954).

GENUS

Contrary to Sabin (1941*a, b*), who suggested grouping the species of the pleuropneumonia group into several genera according to the animal hosts from which they were isolated, we feel that, at least provisionally, only one genus covering all the pleuropneumonia-like organisms should be recognized. The reasons for rejecting Sabin's system of genera based on host-parasite relationships were given in a previous paper (Edward, 1955). Differences in metabolic behaviour of pleuropneumonia-like organisms would seem to provide the soundest basis for distinguishing between genera, and future investigations may provide justification for setting up other genera later. It may be argued that the saprophytic strains should be assigned now to a second genus. The difference in growth requirements that would seem to justify this may, however, be only one of degree, for the organism of goat pleuropneumonia can grow to some extent without serum.

The name for the genus is determined by the first valid name applied to the type species. *Asterococcus mycoides* was the first binomial name given to the organism of bovine pleuropneumonia (Borrel, Dujardin-Beaumetz, Jeantet & Jouan, 1910). While the specific epithet remains valid it was recently recognized by the Editorial Board of the *International Bulletin of Bacteriological Nomenclature and Taxonomy* (1955) that the generic name *Asterococcus* is illegitimate because it is a later homonym of the algal genus *Asterococcus* Scherffel (1908). The Editorial Board suggested two alternatives to replace *Asterococcus*: (1) *Mycoplasma* Nowak (1929), which was the first legitimate generic name given to the organism, and (2) *Borrelomyces* Turner (1935). The latter alternative was suggested on the assumption that it had already come into more general use among bacteriologists. This, however, is certainly not true, and there is nothing to justify the use of this name, which is the fourth in order of priority, in preference for one that has clear priority. Therefore it is proposed that *Mycoplasma* be conserved as the correct and legitimate generic name.

FAMILY

Only one family is recognized for the pleuropneumonia group. Sabin (1941*a, b*) proposed two families: Parasitaceae or Anulomycetaceae and Saprophytaceae or Sapro-mycetaceae. The reasons that were given above for classifying the parasitic and saprophytic species into a common genus may however be even more reasonably applied when speaking of the family.

The appropriate family name under the Rules, if *Mycoplasma* be accepted as the generic name, is Mycoplasmataceae.

ORDER

While Ledingham (1933), on morphological grounds, proposed that the organism of bovine pleuropneumonia and that of agalactia be incorporated in the existing order Actinomycetales, Turner (1935) was the first to realize that the properties of the group were sufficiently distinct to warrant the setting

up of a new order within Schizomycetes. Sabin (1941*a*) believed that the group differed fundamentally from bacteria and therefore assigned it to a separate class, Paramycetes, with one order.

The present authors agree that the organisms of the pleuropneumonia group differ so much from other micro-organisms that they cannot be incorporated into any existing order; they are thus assigned to a separate order. Since the relationship of the organisms to bacteria is uncertain, it seems wise to leave open for the moment the question whether this order should be included within the Schizomycetes. In the 6th edition of *Bergey's Manual of Determinative Bacteriology* (1948) the orders Virales and Rickettsiales are set up as separate groups whose relationships are uncertain. One of us (Edward, 1955) has suggested that the new order should be treated in the same way, as a third supplementary group.

The following names have been proposed for the order to include the organisms of the pleuropneumonia group:

1. Borrelomycetales Turner, 1935.
2. Paramycetales Sabin, 1941*a*.
3. Anulomycetales Sabin, 1941*b*.
4. Pleuropneumoniales Tulasne & Brisou, 1955.
5. Mycoplasmatales Freundt, 1955.
6. Mollicutales Edward, 1955.

Borrelomycetales has priority, and it was for this reason that Freundt (1955) suggested its conservation. However, there appears to be no clear statement in rules of nomenclature that the rule of priority is binding for names of orders. Borrelomycetales itself was derived from *Borrelomyces*, a generic name that is no longer regarded as legitimate; it would seem that there is no longer need to recognize the name derived from it for the order. The name was also rejected by Sabin (1941*a*), since Bordet and not Borrel was the first to describe the morphology of the pleuropneumonia organism. Moreover, *-myces* would possibly seem an unfortunate stem to use if in the future it be decided that the organisms should be placed in a group distinct from the Schizomycetes.

Paramycetales presupposes recognition of a new class, Paramycetes, and Sabin (1941*b*) later recognized its unsuitability as a name for the order and used Anulomycetales derived from *Anulomyces agalaxiae*. This name is also unsuitable since it is based upon an invalid generic name.

The proposal of Tulasne & Brisou (1955) incorporates 'pleuropneumonia' which has provisionally come into general use to provide a comprehensive term for the group. However, it would be unfortunate, as previously pointed out (Edward, 1955), if this recent name, suggested during the course of informal discussion between workers in this field, should be perpetuated by reason of priority alone, since it is based on disease rather than on the organism itself; moreover, the vast majority of species within the order do not cause pleuropneumonia.

The amended Rules of Nomenclature state: 'Rule 2. Names of all taxonomic groups (taxa) above the rank of family are taken preferably from a

combination of characters covering the nature of the taxa as closely as possible, or from a single character of outstanding importance. *Recommendation:* Names of orders and suborders may appropriately be based upon the name of a contained family.' An outstanding and possibly fundamental property of the pleuropneumonia-like organisms is their lack of a rigid cell wall. Mollicutales, based on this property, was therefore suggested as a name of the order. An alternative suggestion is Mycoplasmatales derived from the name of the family and thus fulfilling the recommendation in Rule 2. In this way the introduction of a new name into the nomenclature of pleuropneumonia-like organisms is avoided. This name, too, is based on the plastic nature of the organisms. That Nowak (1929) when proposing *Mycoplasma* as the generic name intended to allude by this to the plasticity of the pleuropneumonia organism appears from his description: 'Le microbe semble, au mois à certains stades de son cycle évolutif, n'être qu'un petit amas de protoplasme à demi liquide, épais, d'une consistance homogène; il n'est donc pas différencié en exoplasme et endoplasme. Cette circonstance semble faciliter son polymorphisme' (p. 1348). Mycoplasmatales, however, is less preferable than Mollicutales as a descriptive term since it suggests a relationship with the fungi. It is proposed that Mollicutales and Mycoplasmatales be submitted for consideration as possible alternative names for the order.

ESTABLISHED SPECIES

The selection of species to be included in the classification has been guided by the principle that for each species which is named and defined there should be kept in collections of type cultures a representative strain upon which the properties of that species have been based. *Asterococcus* I and II (Shoetensack, 1936), *Musculomyces arthrotropicus* and *M. histotropicus* (Sabin, 1941a) set up earlier and based on strains which are no longer in existence have thus been omitted, because they cannot be definitely identified with any new isolates by reference to the original descriptions. Other strains of pleuropneumonia-like organisms that have been reported in recent years from various sources, e.g. from bronchopneumonia of cattle (Carter, 1954) and from the eyes of a chameleon (Klinge, 1954), need further study before they can be named as separate species.

The following tentative list of species is based on the lists previously published by each of us. The few points on which they deviated from each other have been adjusted. A new species, *Mycoplasma hyorhinalis* Switzer (1955), has been added. Strain numbers refer to the collection at the Wellcome Research Laboratories.

ORDER

- Alternative 1. Mycoplasmatales, Freundt, 1955.
- Alternative 2. Mollicutales, Edward, 1955.

FAMILY

Mycoplasmataceae.

GENUS

Mycoplasma, Nowak, 1929.

The type species is *Mycoplasma mycoides* (Borrel *et al.*) Freundt.

Species 1. *Mycoplasma mycoides* (Borrel *et al.* 1910) Freundt.

Subspecies 1. *Mycoplasma mucoides* var. *mycoides*. Organism causing contagious pleuropneumonia of cattle. Representative strain: PG 1.

Subspecies 2. *Mycoplasma mycoides* var. *capri* (Edward, 1954) Freundt. Organism causing contagious pleuropneumonia and other infections in goats. Representative strain: PG 3.

Species 2. *Mycoplasma agalactiae* (Wroblewski, 1931) Freundt. Organism causing contagious agalactia. Representative strain: PG 2.

Species 3. *Mycoplasma bovis* (Freundt, 1950). P strains from cattle (Edward, 1950). Representative strain: PG 11.

Species 4. *Mycoplasma spumans* Edward. α strains from dogs (Edward & Fitzgerald, 1951a). Representative strain: PG 13.

Species 5. *Mycoplasma canis* Edward. β strains from dogs (Edward & Fitzgerald, 1951a). Representative strain: PG 14. (Not *Asterococcus canis* Shoetensack, 1936.)

Species 6. *Mycoplasma maculosum* Edward. γ strains from dogs (Edward & Fitzgerald, 1951a). Representative strain: PG 15.

Species 7. *Mycoplasma pulmonis* (Sabin, 1941a) Freundt. L. 3 strains from rats (Klieneberger, 1938). Representative strain: 'Ash'.

Species 8. *Mycoplasma arthritidis* (Sabin, 1941a) Freundt. L. 4 strains from rats. Representative strain: 'Preston' (PG 6).

Species 9. *Mycoplasma neurolyticum* (Sabin, 1941a) Freundt. Type A and L. 5 strains from mice. Representative strain: L. 5 (PG 28).

Species 10. *Mycoplasma gallinarum* Freundt, 1955. Based on the cultural and biochemical examination of one strain (Edward, 1954). Relationship to strains isolated from chickens and turkeys by American and Canadian workers not yet established. Representative strain: PG 16.

Species 11. *Mycoplasma hominis* Edward, 1955.

Type 1. Human type 1 strains (Nicol & Edward, 1953). Representative strain: PG 21.

Type 2. Human type 2 strains (Nicol & Edward, 1953). Representative strain: PG 27.

Species 12. *Mycoplasma fermentans* Edward, 1955. Human type 3 strains (Nicol & Edward, 1953). Representative strain: PG 18.

Species 13. *Mycoplasma salivarium* Edward, 1955. Human type 4 strains (Nicol & Edward 1953). Representative strain: PG 20.

Species 14. *Mycoplasma laidlawii* (Sabin, 1941a) Freundt. Sewage and other saprophytic strains. Representative strain: Sewage A (Laidlaw & Elford, 1936), (PG 8). The specific epithet, *laidlawii*, suggested by Sabin has been altered to conform with the Rules for Bacteriological Nomenclature.

Species 15. *Mycoplasma hyorhinis* (Switzer, 1955, nov. sp.). Organism causing a generalized infection in swine involving the serous membranes of the thoracic and abdominal cavities (Glasser's disease): Switzer (1953a, b). Representative strain: PG 29.

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Phage Typing of *Salmonella typhimurium*: its Place in Epidemiological and Epizootiological Investigations

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SUMMARY: Two series of adapted phages were compared: the first consisted of phage preparations belonging to three different antigenic groups, the second series was antigenically homogeneous. The two typing schemes based on these phage preparations yielded entirely different groupings of the *Salmonella typhimurium* strains examined. Nevertheless, by each of the schemes it was possible to identify those strains which were related epidemiologically. These findings are compared with those obtained with Lilleengen's series of selected anti-O phages, which are employed in Sweden. The place of phage typing in epidemiological and epizootiological investigations is discussed. It is concluded that it is unnecessary to attempt international standardization of the phage typing of *S. typhimurium* or of any other *Salmonella* species which cause food poisoning in man.

Soon after the introduction of phage typing of *Salmonella typhi* (Craigie & Yen, 1938*a, b*) and of *S. paratyphi-B* (Felix & Callow, 1943) attempts were made to develop a similar typing procedure for *S. typhimurium*, the most common cause of food-poisoning in man. A typing scheme evolved by Felix & Callow has been in use in Great Britain for the past ten years, though it has not yet been published in detail (Felix & Callow, 1943; Felix, 1951). An entirely different typing scheme, devised independently by Lilleengen (1947, 1948), has been employed in Sweden. It was originally thought desirable to standardize the typing procedure for *S. typhimurium* by international agreement in a way similar to that adopted for the typing of typhoid and paratyphoid-B bacilli (Craigie & Felix, 1947; Felix, 1951, 1953*b*). The present paper shows that this is unnecessary. The investigations on which this conclusion is based were carried out at the Central Enteric Reference Laboratory of the Public Health Laboratory Service of England and Wales (Medical Research Council), in collaboration with Miss B. R. Callow. Only those results are summarized here which help to make clear the reasons for reaching this conclusion. A description of the technical details of the experiments will be published elsewhere by Miss B. R. Callow.

METHODS

Typing technique. The technique described by Craigie & Felix (1947) was followed throughout.

Media. Broth and agar media used for the routine typing of strains were made with Bacto dehydrated nutrient broth (20 g./l.), according to the formula recommended by Craigie & Felix (1947). These media are referred to as Difco broth and Difco agar.

For the propagation and testing of phage preparations tryptic digest broth

and agar were employed. It was stated previously, in connexion with work on *Salmonella paratyphi-B* phages (Felix & Callow, 1951), that trypsin-digest broth and agar proved superior to Difco broth and agar, although the latter media are preferable for work with *S. typhi* phages. *S. typhimurium* phages behave in the same way as those of *S. paratyphi-B*. The reason for this puzzling difference between members of the *Salmonella* group is unknown, but the finding is of considerable practical importance.

The tryptic digest of meat should be made from not less than 500 g. fresh meat/l. and should always be prepared in large batches. Each successive batch should be compared with the previous one by parallel tests of selected strains and phage preparations, and unsatisfactory batches should be rejected. The far-reaching effects which the quality of the agar medium may have on the size of the plaques produced have been emphasized in previous papers (Craigie & Felix, 1947; Felix & Anderson, 1951). Commercial peptone should not be added since it does not improve a good meat-digest as a medium for the particular purpose under discussion.

Temperature of incubation and reading of tests. The plates were incubated at 38.5°, in preference to the customary incubation at 37.5°. The first reading was taken after incubation for 4½ hr. and a second reading after incubation for a further 3½ hr. Thirty minutes were always allowed for warming up the plates, i.e. the first 30 min. were not counted. There is very little difference between readings taken after interrupted or non-interrupted incubation for 8 hr. Readings taken after longer periods of incubation are less satisfactory because of the heavy secondary growth of resistant organisms.

Neutralization tests. The technique used was that previously employed in experiments with *Salmonella paratyphi-B* phages (Felix & Callow, 1951) and with *S. typhi* phages (Anderson & Felix, 1953). The titre was arbitrarily fixed as being the dilution of serum which neutralized approximately 90% of the homologous phage particles. Great importance was attached to the inclusion in the tests of pre-immunization samples of serum from each individual rabbit, to serve as controls of the corresponding post-immunization sera.

During the early part of this investigation the phage/serum mixtures were incubated at 38.5° for 3 hr. and then kept in the refrigerator overnight at +2°. Afterwards it was found that neutralization was equally effective when the phage/serum mixtures were stored in the refrigerator for 16–20 hr. at +2° without having been incubated at all. This procedure proved particularly useful in experiments with low-titre preparations of natural phages. These could thus be employed in the neutralization test without having been completely sterilized, a task that is often difficult to accomplish with these phages.

RESULTS

The Felix & Callow typing scheme for Salmonella typhimurium

Felix & Callow (1943) found that *Salmonella typhimurium* was not lysed by any of the four paratyphoid-B typing phages known at that time, but that it was sensitive to its own, apparently specific, phages. The first typing scheme

for *S. paratyphi-B*, described in Table 1 in their 1943 paper, also showed the lytic reactions produced by the first typing phage of *S. typhimurium* which had been grown from a specimen of faeces of a patient suffering from food poisoning. This phage was found to act selectively on the strain of *S. typhimurium* isolated from the same faecal specimen.

Many strains of *Salmonella typhimurium* isolated from different outbreaks of food poisoning were examined with a view to adapting the first specific *S. typhimurium* phage. In a few instances adapted phage preparations were obtained, which possessed properties similar to those of adapted typhoid Vi phages (Craigie & Yen, 1938*a, b*). However, in many other instances all attempts at adaptation of the first specific *S. typhimurium* phage failed.

On the other hand, it was found that phage 3b of *Salmonella paratyphi-B* often produced isolated plaques on strains of *S. typhimurium* and that these yielded phage preparations adapted to some of the strains refractory to the original specific *S. typhimurium* phage. Other phage preparations which showed selective lytic action were added to the set of typing phages and a typing scheme shown in Table 1 was thus evolved.

Table 1. *Typing scheme of Felix & Callow for Salmonella typhimurium*

Type strains		First series of typing phages at routine test dilutions											
Group	Type	1	1a	1b	2	2a	2b	2c	2d	3	3a	3b	4
I	1	OL	OL	<CL	CL	CL	OL	CL	OL	OL	OL	OL	±
	1a	—	OL	<CL	CL	<CL	OL	<CL	OL	OL	OL	OL	—
	1b	—	—	<CL	CL	CL	OL	<CL	OL	<CL	<CL	<CL	—
II	2	—	—	—	CL	CL	—	CL	—	—	—	—	—
	2a	—	—	—	—	CL	<CL	CL	<CL	—	—	—	—
	2b	—	—	—	—	—	OL	—	OL	—	—	—	—
	2c	—	—	—	<CL	<CL	<CL	<CL	<CL	—	—	—	—
	2d	—	—	—	±	±	±	±	<CL	—	—	—	—
III	3	—	—	++	—	—	—	—	—	OL	++	±	—
	3a	—	—	—	—	—	+++	—	SCL	+	<CL	±	—
	3b	—	OL	<CL	—	+	OL	—	OL	OL	OL	OL	—
IV	4	—	—	—	±	±	—	±	±	—	—	—	<CL

CL=confluent lysis; <CL=almost confluent lysis; SCL=semi-confluent lysis; OL=opaque lysis giving island effect; ±, +, ++, +++=increasingly numerous plaques; —=no plaques. Homologous and group reactions are shown in heavy type.

The arrangement of the types shown in Table 1 is that evolved in the course of the routine application of the typing phages as they became available. The twelve types and the corresponding typing phages are grouped in four groups. Group II contains the first type referred to above (Felix & Callow, 1943). The specific phage which in 1943 was labelled phage 1 is now designated as phage 2. The phage preparations 2a and 2c are adaptations of phage 2. Phages 2b and 2d are descendants of a phage carried by a rough culture of *Salmonella enteritidis*. The three typing phages in group I (phages 1, 1a and 1b) are adaptations of phage 3b of *S. paratyphi-B*. The origin of the remaining four phages will be discussed later in connexion with their antigenic properties as revealed by cross-neutralization tests.

The specificity of the adapted typing phages of *Salmonella paratyphi-B* and *S. typhimurium* at first appeared to be as great as that of the typhoid Vi phages. The term 'specific Vi phages' was, therefore, employed in earlier papers (Felix & Callow, 1943, 1951; Felix, 1951) on the assumption that these phages acted on the labile Vi antigen which *S. paratyphi-B* shares with *S. typhimurium*, i.e. the BVi antigen which is identical with O-factor V (five) of the Kauffmann-White scheme. However, it was later found that all the paratyphoid-B and *S. typhimurium* typing phages also lysed strains of *S. gallinarum* and *S. pullorum*, organisms which do not contain the BVi antigen. The erroneous designation 'BVi' phages was therefore discontinued (Felix, 1953*b*). The same conclusion regarding the paratyphoid-B typing phages was reached independently and almost simultaneously by Cherry, Davis & Edwards (1953) and by Sicca & D'Amelio (1953). The phenomenon of phage adaptability is evidently not restricted to the special typhoid Vi phages in which it was first observed by Craigie & Yen (1938*a*) but is one of much wider application (e.g. Luria & Human, 1952; Bertani & Weigle, 1953; Ralston & Krueger, 1954). On the other hand, it is obvious that these findings about adapted phages of *S. paratyphi-B* and *S. typhimurium* have nothing to do with the question whether the labile somatic antigen common to these two organisms should be designated as a Vi antigen (Felix, 1952*a, b*) or as a K antigen (Kauffmann, 1954).

Constancy of Salmonella typhimurium phage types and their epidemiological significance

Although the typing scheme shown in Table 1 was evolved in an empirical manner, it was nevertheless found that the constancy and epidemiological significance of the types so defined were of the same high order as those of typhoid and paratyphoid-B phage types. Many outbreaks of food-poisoning in Great Britain were investigated during the past ten years by this technique, and the usefulness and reliability of the method were established beyond doubt. A few of these outbreaks have been described in detail in published reports; the relevant data are summarized in Table 2. The designation 'group II' was originally employed for strains which subsequently were assigned to one of the five phage types of group II (see paper by Blaxland & Blowers, 1951, listed in Table 2).

A striking example of the stability of the phage types determined by the scheme shown in Table 1 was recently provided during the examination of a number of non-motile O variants of *Salmonella typhimurium* and *S. paratyphi-B*, which were employed by Stocker, Zinder & Lederberg (1953) in experiments on the transduction of H antigens. Identical patterns of lytic reactions were recorded in 1953 with cultures of two *S. typhimurium* strains which had been kept since 1929 in the National Collection of Type Cultures (in dried form since 1939) and with cultures which had been in continuous use and had been subcultured very many times during the intervening years.

Table 2. *List of published reports of phage-typed outbreaks in England and Wales due to infection with Salmonella typhimurium*

Outbreaks					
Food poisoning					
Year	No. of persons affected	Vehicle of infection	Infection in animals	Phage type	Reference
1944	1 (fatal)	Duck egg	Flock of ducks	2	Gordon & Buxton (1945); Gillespie (1946)
1947	43	Dried egg	.	1	Cook & de Costobadie (1947)
1949	28	Duck eggs	Flock of ducks	group II	Blaxland & Blowers (1951)
1949	1 (fatal)	Duck egg	Flock of ducks	2	Miller (1952)
1949	3 (1 fatal)	Duck eggs	Flocks of ducks	4	
1949	224	Cream pastries	.	4	Kwantes (1952)
1950	1	Duck egg	Drake	4	Eedy (1950)
1951	.	.	Herd of dairy cows, pigs, ducks and hens	1	Sellers & Sinclair (1953)
1953	252	Milk	Dairy cow	1a	Norton & Armstrong (1954)
1954	7	Direct transmission from turkeys	Flock of turkeys	1a	Pereira & Blaxland (1955)

Table 3. *Serological groups of typing phages of Salmonella typhimurium*
First series of twelve typing phages of Felix & Callow, 1942-46.

Group 1		Group 2		Group 3
Phage preparation derived from		Phage preparation derived from		
Phage 1 } Phage 1a } Phage 1b }	Phage 3b of <i>S. paratyphi-B</i>	Phage 2 { Faecal specimen containing <i>S. typhimurium</i> strain 668		Phage 4 Probably derived from a 'natural' phage of <i>S. typhimurium</i>
Phage 2b } Phage 2d }	Phage carried by rough strain of <i>S. enteritidis</i>	Phage 2a } Phage 2c }	Phage 2	
Phage 3 } Phage 3a } Phage 3b }	Phage 2a			

Antigenic properties of the twelve typing phages

As a preliminary to the intended international standardization of the typing procedure, the twelve typing phage preparations were examined for their antigenic properties. The results of numerous cross-neutralization tests, carried out with a representative series of antiphage sera, are summarized in Table 3. This shows that cross-neutralization tests subdivided the twelve typing phages into three serological groups and that these groups did not coincide with those

shown in Table 1. The three phages in serological group 2 showed complete cross-neutralization, as was to be expected because of their common origin; so also did phages 1, 1a and 1b. However, the remaining five typing phages in serological group 1 did not behave according to expectation. If phages 3, 3a and 3b were genuine adaptations of phage 2a, as they were presumed to be in view of the procedure followed in their preparation, they should have been serologically identical with their parent phage 2a. Similarly, the fact that phages 2b and 2d belonged to group 1 was also difficult to reconcile with their antecedents. Thus the position was similar to that previously met in work on the adapted typing phages of *Salmonella paratyphi-B* (Felix & Callow, 1951) and the same alternative explanations offered themselves, namely:

(a) The apparently anomalous serological reactions obtained with phages 3, 3a and 3b might have been due to contamination at some stage during the isolation and propagation of these phages. In spite of meticulous observance of the necessary precautions it is impossible to exclude with certainty airborne contamination in a laboratory where bacteriophage preparations are being handled, or the possibility of one of the bacterial strains having had contact with a particular phage before the culture reached the laboratory.

(b) The observed change in the serological properties of phage 2a, from which phages 3, 3a and 3b were derived, might represent another instance of antigenic variation occurring in a bacteriophage during its adaptation to a bacterial cell of a different phage type. It was stated in a previous paper that since it was well established that adaptation led to variation in lytic activity of the phage particle, there was no reason for rejecting *a priori* the possibility of variation in its antigenic constitution. This view certainly appears to be much less unorthodox now than it was at the time it was first put forward in connexion with the study of adapted phages of *Salmonella paratyphi-B* (Felix & Callow, 1951). Recent work of Scholtens (1952, 1955*a, b*) on *S. paratyphi-B* phages generated in mixed cultures of different phage types lends strong support to the thesis of antigenic variation in bacteriophage as part of the phenomenon of phage adaptation. Whether the phenomenon is adequately described as recombination of genetic elements of the bacterial cell (Scholtens, 1952) need not be discussed here.

Experiments with a different set of adapted typing phages

In view of the antigenic heterogeneity of the *Salmonella typhimurium* phages hitherto employed, an attempt was made to replace the set of typing phages by one conforming strictly to the rule followed in the typing of *S. typhi*, namely, that all the phage preparations should be derived by adaptation from one strain of bacteriophage and that all should be neutralized by antiserum prepared from that phage (Craigie & Felix, 1947).

The original specific *Salmonella typhimurium* phage (phage 2, no. 668/668) could not be used for this purpose because it was known from earlier work that it was impossible to adapt that phage to strains belonging to types 1, 1a and 1b, all of which are common in Great Britain and therefore of practical importance.

Table 4. *Grouping of selected twenty-three phage types and subtypes of Salmonella typhimurium by first series of typing phages*

Routine test dilutions of first series of typing phages (Felix & Callow, 1942-46)														
Type strains														
Types and subtypes	Original designation (Type)	Strain no.	1	1a	1b	2	2a	2b	2c	2d	3	3a	3b	4
1	1	307	OL*	419	1414	668	154	392	590	1272	298	736	435	1166
1a	1a	5000	OL*	4000	3000	100,000	10,000	3000	1000	1000	1000	1000	1000	800
1a var. 1	1a		-	OL*	<CL	CL	CL	OL	CL	OL	OL	OL	OL	±
1a var. 4	3b		-	OL*	<CL	CL	CL	OL	CL	OL	OL	OL	OL	-
1b	.		-	OL	<CL	-	±	OL	-	OL	OL	OL	OL*	-
1b var. 3	1b		-	++	<CL*	CL	CL	OL	<CL	OL	<CL	<CL	<CL	-
2 var. 1	3		-	-	++	-	-	-	-	-	OL*	±	±	-
2 var. 2	.		-	-	-	CL	CL	-	CL	-	-	-	-	-
2 var. 3	2		-	-	-	CL*	CL	-	CL	-	-	-	-	-
2 var. 4	2		-	-	-	CL	CL	-	CL	-	-	-	-	-
2a	.		-	-	-	-	CL	-	CL	<CL	-	-	-	-
2a var. 1	2a	154	-	-	-	-	CL*	<CL	CL	<CL	-	-	-	-
2b var. 1	.	1899	-	-	-	-	-	OL	-	OL	-	-	-	-
2b var. 2	2b	2540	-	-	-	-	-	OL*	-	OL	-	-	-	-
2b var. 3	.	2200	-	-	-	-	-	-	-	OL	-	-	-	-
2c var. 1	.	1939	-	-	-	-	-	OL	-	OL	-	-	-	-
2c var. 2	2c	591	-	-	-	++	<CL	<CL	<CL	<CL	-	-	-	-
2c var. 3	.	1957	-	-	-	<CL	<CL	<CL*	<CL	<CL	-	-	-	-
2c var. 4	.	1184	-	-	-	<CL	<CL	<CL	<CL	<CL	-	-	-	-
2d var. 1	2d	1272	-	-	-	<CL	<CL	<CL	<CL	<CL	-	-	-	-
2d var. 2	.	2471	-	-	-	±	±	±	±	<CL*	-	-	-	-
2d var. 3	.	1431	-	-	-	-	±	±	±	<CL	-	-	-	-
3a	3a	736	-	-	-	-	-	-	-	<CL	-	<CL*	±	-
4	4	1166	-	-	-	±	±	±	±	<CL	+	±	±	<CL*

* Indicates homologous phage preparation and type strain.
Group reactions are shown in heavy type.

Table 5A. *Grouping of selected twenty-three phage types and subtypes of Salmonella typhimurium by second series of typing phages*

Test dilutions of typing phages													
Type strains			Second series (Felix & Callow, 1950-54)										
			First series										
Types and subtypes	Original designation (Type)	Strain no.	1	1a	1b	1a var. 4	1b var. 3	2 var. 1	2 var. 3	2a	2c var. 1	2c var. 2	2d var. 1
1	1	307	OL*	OL	<CL	+	SCL	SCL	OL	OL	OL	OL	OL
1a	1a	419	-	OL*	<CL	OL	OL	SCL	OL	OL	OL	OL	-
1a var. 1	3b	435	-	OL	<CL	OL	SCL	+	SCL	SCL	SCL	+	-
1a var. 4	.	1505	-	++	<CL*	SCL*	-	±	SCL	SCL	±	±	-
1b	1b	1414	-	-	<CL*	-	OL	-	-	+	SCL	OL	-
1b var. 3	3	298	-	-	++	+	SCL*	-	-	+	+	+	-
2 var. 1	.	2317	-	-	++	SCL	-	OL*	OL	-	+	+	-
2 var. 2	.	1221	-	-	-	-	-	+	+	-	-	-	-
2 var. 3	2	668	-	-	-	SCL	-	+	OL*	-	-	-	-
2 var. 4	.	944	-	-	-	-	-	-	-	-	-	-	-
2a	2a	154	-	-	-	SCL	-	+	-	-	-	-	-
2b var. 1	.	1899	-	-	-	SCL	-	+	<OL	<OL*	<OL	+	-
2b var. 2	2b	2540	-	-	-	-	-	-	-	-	-	+	+
2b var. 3	.	2200	-	-	-	-	-	-	-	-	-	SCL	-
2c var. 1	.	1939	-	-	-	-	-	-	+	+	+	SCL	-
2c var. 2	2c	591	-	-	-	-	-	-	-	-	<OL*	-	-
2c var. 3	.	1957	-	-	-	-	-	-	-	-	-	OL*	-
2c var. 4	.	1184	-	-	-	-	-	-	-	-	-	OL	-
2d var. 1	2d	1272	-	-	-	-	-	-	-	-	-	OL	-
2d var. 2	.	2471	-	-	-	-	-	-	-	-	-	-	SCL*
2d var. 3	.	1431	-	-	-	-	-	-	-	-	-	-	-
3a	3a	736	-	-	-	-	-	-	-	-	-	-	-
4	4	1166	-	-	-	+	+	OL	OL	-	-	-	-

* Indicates homologous phage preparation and type strain.
Group reactions are shown in heavy type.

Phage 3b of *S. paratyphi-B* was chosen as the one from which the new series of adapted phages was to be built up. The reasons for this choice were:

(a) Eight of the twelve phages of the old series were known to be serologically identical with phage 3b of *Salmonella paratyphi-B* (see Table 3). The first three phages of this series had been derived from phage 3b of *S. paratyphi-B* by the customary technique and were therefore to be accepted as genuine adaptations of that phage.

(b) Since it was known that every strain of *Salmonella typhimurium* is lysogenic (Boyd, 1950; Callow, to be published), it was important to ascertain that the phage chosen for adaptation did not occur, or occurred only very rarely, as a natural phage of strains of *S. typhimurium*. Otherwise, adaptation to these strains would not take place because of insusceptibility due to the presence in the bacterial cell of the identical natural phage. Phage 3b of *S. paratyphi-B* is the most common natural phage of strains of *S. paratyphi-B* (Felix & Callow, 1951; Scholtens, 1952, 1955*a, b*) but, fortunately, it does not seem to occur at all among the natural phages of *S. typhimurium*. Extensive investigations of the natural phages of strains of *S. typhimurium*, representing all the known phage types and subtypes of this organism, were carried out by Miss B. R. Callow and will be reported by her elsewhere.

It was known from observations made in the course of routine typing that most of the types listed in Table 1 could be further subdivided according to variations in the pattern of the lytic reactions displayed by certain strains, or groups of strains, isolated from different foci of infection. The different patterns of reaction to the typing phages were often correlated with the presence in the culture of one, two or more different natural phages, in the same way as was shown in earlier work on *Salmonella paratyphi-B* (Nicolle, Hamon & Edlinger, 1951; Hamon & Nicolle, 1951; Scholtens, 1950, 1952; Felix & Callow, 1951). In other instances differences in the content of the cultures of certain natural phages were observed, but these were not reflected by the pattern of reactions to the typing phages. On the basis of these two criteria more than forty types and subtypes of *S. typhimurium* could be distinguished (Callow, to be published), and of these twenty-three were selected for the comparative tests shown in Tables 4, 5A and 5B.

The typing phages listed in Table 4 are identical with those used throughout the work described in the preceding sections of this paper. Table 4 thus represents an extended version of the original typing scheme shown in Table 1, enlarged to include a number of additional types or subtypes and to give the particulars necessary for the specification of the type strains and typing phages.

The second set of typing phages listed in Tables 5A and 5B consisted only of genuine adaptations of phage 3b of *Salmonella paratyphi-B*, all of which were fully neutralized by antisera prepared against that phage. The three old series phages 1, 1a and 1b were accepted for the second series, since they conformed to the criteria originally formulated. The remaining eight phage preparations were adaptations of phage 3b of *S. paratyphi-B* to the type cultures of selected types or subtypes of *S. typhimurium*. Some of the adapted phages were

Table 5B. *Alternative grouping of selected twenty-three phage types and subtypes of Salmonella typhimurium by second series of typing phages*

Type strains			Test dilutions of typing phages										
			First series			Second series (Felix & Callow, 1950-54)							
Types and subtypes	Original designation (Type)	Strain no.	1	1a	1b	1b var. 3	2a	2c var. 1	2c var. 2	2d var. 1	1a var. 4	2 var. 1	2 var. 3
1	1	307	5000	419	1414	298	154	1939	591	1272	1505	2317	668
1a	1a	307	OL*	OL	<CL	SCL	OL	OL	OL	OL	++	SCL	OL
1a var. 1	3b	435	—	OL*	<CL	OL	OL	OL	++	—	OL	SCL	OL
1a var. 4	.	1505	—	OL	<CL	SCL	SCL	SCL	++	—	OL	++	SCL
1b	1b	1414	—	++	—	—	SCL	±	±	—	SCL*	±	SCL
1b var. 3	3	298	—	++	<CL*	OL*	++	++	OL	—	—	—	—
2a	2a	154	—	—	++	SCL*	<OL*	<OL*	±	—	—	—	—
2c var. 1	.	1939	—	—	—	—	++	<OL*	—	—	SCL	++	<OL
2c var. 2	2c	591	—	—	—	—	—	—	OL*	—	—	—	—
2c var. 3	.	1957	—	—	—	—	—	—	OL	—	—	—	—
2c var. 4	.	1184	—	—	—	—	—	—	OL	—	—	—	—
2b var. 1	.	1899	—	—	—	—	—	—	++	—	—	—	—
2b var. 2	2b	2540	—	—	—	—	—	—	SCL	—	—	—	—
2b var. 3	.	2200	—	—	—	—	—	—	SCL	—	—	—	—
2d var. 1	2d	1272	—	—	—	—	—	—	—	SCL*	—	—	—
2 var. 1	.	2317	—	—	—	—	—	—	—	—	SCL	—	—
2 var. 3	2	668	—	—	—	—	—	—	—	—	—	—	—
4	4	1166	—	—	—	—	—	—	—	—	—	—	—
2 var. 2	.	1221	—	—	—	—	—	—	—	—	SCL	OL*	OL
2 var. 4	.	944	—	—	—	—	—	—	—	—	SCL	++	OL*
2d var. 2	.	2471	—	—	—	—	—	—	—	—	++	OL	—
2d var. 3	.	1431	—	—	—	—	—	—	—	—	—	—	—
3a	3a	736	—	—	—	—	—	—	—	—	—	—	—

* Indicates homologous phage preparation and type strain.
Group reactions are shown in heavy type.

derived directly from the original phage 3b of *S. paratyphi-B*, others from one of its well-authenticated descendants. The preparation, purification and testing of these adapted phages is a difficult and time-consuming process, the details of which will be described elsewhere by Miss B. R. Callow.

A comparison of Tables 4 and 5A shows that the additional eight adapted phages of the second series produced lytic reactions which cut across the groups established by means of the first series of phages. Particular interest attaches to the differences in the lytic spectra of those phages which had been adapted to, and propagated on, the same type culture. There were four such pairs of phage preparations in the two series. Only one of these pairs, i.e. the two phages adapted to strain 298, showed identical lytic spectra, and in this case both phages belonged to the same serological group (group 1). The other three pairs of phage preparations, i.e. those adapted to the type cultures 668, 154 and 1272, showed widely differing lytic spectra. This was not surprising in the case of the two pairs of phages numbered 668 and 154 belonging to two different serological groups (groups 1 and 2). On the other hand, the striking difference in the range of lytic activity of the two phages adapted to strain 1272 came as something of a surprise, since both gave the clear-cut reactions of serological group 1.

Table 5A also shows that five of the type strains that were readily typed by means of the first series of phages were resistant to all the phages in the second series and thus remained 'untypable'. By arranging the type strains and typing phages of the second series in a different order, as listed in Table 5B, an alternative grouping of the selected twenty-three phage types and subtypes was obtained. This grouping was totally different from that shown in Table 4. Nevertheless, strains which were related epidemiologically could be identified as such, whether they were tested against the first series of typing phages or against the second. That is to say, both typing schemes gave results that were in accord with the known epidemiological data.

DISCUSSION

In a previous paper (Felix, 1951) it was stated that the typing of strains of *Salmonella typhimurium* according to the scheme described by Lilleengen (1947, 1948) is not of the same epidemiological significance as that of typing with the adapted phages employed by Felix & Callow. This conclusion was reached on the basis of comparative tests carried out in 1948 in collaboration with Professor Lilleengen during his visit to the writer's laboratory at Colindale. By means of Lilleengen's set of twelve typing phages, which were designated as anti-O phages because they lysed many diverse *Salmonella* species, strains of *S. typhimurium* were divided into twenty-four different types. However, this grouping and that according to the Felix & Callow scheme cut across one another. Since experience of the latter technique had shown that the typing results invariably agreed with the epidemiological findings, the conclusion appeared to be logical that the entirely different grouping of the strains resulting from tests with Lilleengen's phages could not

be of the same significance. This conclusion is now shown to have been premature.

The present paper shows that it is possible to identify strains isolated from the same focus of infection by means of two different series of adapted phages which possess different lytic spectra and yield entirely different groupings of the strains. Furthermore, the specifically adapted *Salmonella typhimurium* phages used by Felix & Callow are now known to be O phages, like those of Lilleengen's series which had been collected in a more random fashion.

Recent work on 'auxiliary' typing of *Salmonella typhi* and *S. paratyphi-B* has provided other examples of the successful application as typing phages of preparations derived from natural phages which had not been subjected to any elaborate process of adaptation (Nicolle, Pavlatou & Diverneau, 1953, 1954; Nicolle & Diverneau, 1955; Nicolle, Hamon & Edlinger, 1953; Scholtens, 1950, 1952, 1955*a, b*). These findings are in keeping with the view that the multiplicity of phage-systems applicable to any bacterial species is correlated with the multiplicity of enzyme-systems at work in that species (Felix, 1949, 1953*a*). The facts established in connexion with the work on the natural phages of the various *Salmonella* species are considered to provide additional evidence in favour of the writer's view that bacteriophages are part of the genetic make-up of the bacterium, not parasites of extrinsic origin as postulated for animal viruses. A similar conclusion was arrived at by Scholtens (1952, 1955*b, c*).

It has long been recognized that the practical value of the bacteriophage typing of *Salmonella typhimurium* is not as great as that of typhoid or paratyphoid-B phage typing (Felix, 1951). Chronic carriers of *S. typhimurium* are extremely rare and play a negligible role in the spread of the infection, and it is in the detection of the chronic carrier that the bacteriophage technique renders the greatest service. One of the main functions of a phage-typing centre, where strains of *S. typhi* or *S. paratyphi-B* are being examined, is to maintain a register of cases and carriers, arranged according to the phage type of the bacteria with which each of the patients had been, or is, infected. It is not feasible to use similar information in the laboratory control of *S. typhimurium* infections, since these occur not only in man but in many domestic animals, including mice and rats, which serve as reservoirs of the infection. For this reason the typing of *S. typhimurium* strains has not been adopted as a routine in every outbreak or sporadic case, as it has been in cases of typhoid and paratyphoid-B fever. Evidently, the spending of time and effort on such routine examination would be unwarranted.

On the other hand, circumstances may arise in the course of the investigation of an outbreak of *Salmonella typhimurium* infection in man or in animals which compel the epidemiologist or epizootiologist, to seek guidance, or final confirmation of his findings, from the phage typing of the cultures isolated from patients, foodstuffs, flocks of poultry or animals, etc. When a careful investigation is conducted and its successful conclusion is considered to depend on the application of the phage-typing method, an adequate number of cultures should be examined. All that is needed in such cases is to establish identity or non-

identity of the cultures under examination. It is clear that this may be readily accomplished by means of a suitable set of phage preparations without following an internationally agreed standard procedure.

The usefulness in certain circumstances of the phage typing of *Salmonella typhimurium* is exemplified by the published reports of a number of field investigations conducted in Great Britain during 1944-54 (see Table 2). In Sweden, where an outbreak of food poisoning involving many thousands of persons occurred in 1953, typing with Lilleengen's phages has been employed for the past few years at the Royal Veterinary College, Stockholm (Lilleengen, personal communication) and at the State Bacteriological Laboratory, Stockholm (Lundbäck, personal communication). According to these workers the results obtained have greatly facilitated the follow-up of the very large outbreak in 1953 and also the investigation of a number of small outbreaks.

The adapted phages of the first and second series employed by Felix & Callow have an important advantage over Lilleengen's less specific phage preparations. The latter give reliable results only in test dilutions which have to be determined, and often re-adjusted, with utmost accuracy. Lilleengen found it necessary to resort to titration of his phage preparations in seven dilutions intermediate between the ten-fold stock dilutions (Lilleengen, 1948, p. 46).

The types and subtypes described in this paper represent strains isolated in Great Britain. Their frequency distribution, and the proportion of indigenous strains which are 'untypable', are unknown, because there are no adequate data about the incidence of *Salmonella typhimurium* infection in man or in domestic animals. When a small number of *S. typhimurium* strains isolated in the Suez Canal Zone in 1953 was received for typing (from Colonel M. H. P. Sayers, Fayid) several of the strains were found to represent further types or subtypes, and others were untypable by the first series of typing phages. This indicates the very great number of different phage types and subtypes of *S. typhimurium* which exist in various parts of the world. Since the three different typing schemes discussed in this paper all give results which are valid from the epidemiological point of view, any of these schemes may serve as the starting-point from which an extended scheme may be developed to meet local needs. Contrary to the original plan (Craigie & Felix, 1947; Felix, 1953*b*) it is, therefore, now considered unnecessary to attempt international standardization of the phage typing of *S. typhimurium* or of any other *Salmonella* species causing food poisoning in man (Felix, 1955).

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Isolation of High Acid-yielding Mutants of *Aspergillus niger* by a Paper Culture Selection Technique

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SUMMARY: A method for isolating and identifying high acid-yielding mutants of *Aspergillus niger* is described. This involves cultivation of the organisms on absorbent paper soaked in an indicator medium. The advantages of the technique and the criteria used for selecting biochemically interesting mutants are briefly described. The greater acid production of mutants selected by paper culture has been confirmed by comparing yields of citric acid in surface and submerged fermentations with those given by the wild type strain.

The selection of acid-producing fungi described by Foster & Davis (1949) and modified by Quilico, Panizzi & Visconti (1949) has been used frequently for isolating fungi showing marked differences in acid production. Their methods of cultivation on indicator media are unsatisfactory for selection of colonies which produce large amounts of acid because of the extensive diffusion of the acid zones around the colonies. With such strains large quantities of media are required since each colony to be screened would have to be tested on a separate agar plate, and there is no simple means of determining whether such colonies arise from one or more spores.

METHODS

The irradiation techniques used, and a description of the mutant strains isolated by the technique to be described, are given in the following paper (Gardner, James & Rubbo, 1956).

Paper culture selection technique

The method depends on the ability of *Aspergillus niger* to grow as compact discrete colonies surrounded by clearly defined acid zones on absorbent paper previously soaked in liquid culture medium. There are a number of ways of carrying out this paper culture technique, and these can be modified to suit the purpose of the experiment. So far the following procedure has been used.

Circular sheets of Eucalypt viscose pulp, 1 mm. thick and 10 in. in diameter were marked with pencil in fifty 1 in. squares and soaked in liquid medium (see below). The moistened paper was supported at four points in a 10 in. Petri dish containing 5 ml. 20% (v/v) glycerol in water to prevent drying of the paper during incubation. The dish and its contents were autoclaved at 116° (10 lb./sq.in. pressure) for 15 min. The medium contained (g./l.): cane molasses (56%, w/v, sucrose), 40; KH_2PO_4 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; NH_4NO_3 , 2.5. To every 100 ml. of this medium (adjusted to pH 5.0) 10 ml. of bromocresol green

(0.4 %, w/v, in ethanol) were added. With the molasses concentration used, the colour change from green to yellow could be clearly seen.

The sterile paper was inoculated by lightly touching it with a fine dropping pipette containing the appropriately diluted spore suspension. The pipette was calibrated to deliver approximately 4000 vol./ml., each volume being that amount of fluid sufficient to cause a transient blanching of a 1 mm. area of the indicator medium. After a little practice in timing the length of contact between the pipette and paper these volumes could be accurately delivered.

Dilution of spore suspension

In order to select mutants according to the size of the acid zones they produced on the paper culture it was important to know that the colonies arose from single spores. Confirmation of single spore implantation on the paper by direct microscopy was impossible, so an indirect method had to be devised. This was done by checking the viable counts on agar and paper culture against the direct spore count in the haemocytometer on three spore suspensions. The results (Table 1) indicate that the viable count by paper culture closely paralleled the beer wort agar plate count and both were in fair agreement with the haemocytometer count. Thus, by diluting the spore suspension according to its microscopic count, a fairly reliable means of ensuring single spore inoculation was obtained.

Table 1. *Comparison of viable counts of Aspergillus niger spores with direct microscopic count*

Sample	No. of spores/ml. $\times 10^4$		
	Haemo- cytometer	Agar plate	Paper culture
A	200	140	140
B	100	70	90
C	50	30	60

Two further checks were introduced. Fisher (1950) showed that the distribution of cells (spores in the present instance) among a number of samples follows the Poisson formula

$$P_x = \frac{m^x}{x!} e^{-m},$$

where P_x is the proportion of samples containing x spores, when the average number of spores/unit volume is m . As the colonies which develop from more than one spore are irregular in size and shape, it is possible to apply this formula to spores cultivated on paper. Close agreement between calculated and observed findings was reached, as shown in Table 2. For instance, when the average number of spores/unit volume (i.e. per drop) was adjusted by haemocytometer count to 0.3, 85 % of the colonies had regular size and shape and hence arose from single spores. The calculated figure was 86 %.

Thus, the irradiated spore suspension was diluted to contain 1500 viable spores/ml. (calculated from the killing curve) for a pipette calibrated to deliver

4000 drops/ml., i.e. 0.3 spores/drop. The paper was inoculated by lightly touching the centre of each marked square with the pipette, incubated at 28°, and examined each day for 6 days.

Table 2. *Comparison between numbers of calculated and observed colonies arising from single spores on paper culture*

No. of spores/ unit vol.	Percentage inoculations of unit volumes showing colonies		Percentage of colonies arising from single spores	
	Calculated	Observed	Calculated	Observed
0.3	26	Av. 28 (31, 25, 30, 26)	86	Av. 85 (84, 85, 87, 85)

Plate 1, fig. 1, shows the paper culture dish and Pl. 1, fig. 2, the colonies of selected mutants after 4 days of incubation at 28°. The colonies which developed on the paper were like those on agar with respect to colour, sporulation and aerial growth. The acid-yielding capacity of different colonies was determined by dividing the diameter of the acid zone by the diameter of the colony, and the figure so obtained was called the 'acid unitage'. In this way it was possible to compare, in a semi-quantitative way, acid production by strains with widely differing growth rates (see Table 3). The acid unitage was estimated for each colony and colonies which gave higher values than the parent strain were transferred to beer wort agar slopes for subsequent tests of purity and for screening of fermentation ability.

Screening of mutants

Preliminary fermentation tests with selected strains were carried out by the surface culture method, with cane molasses as the substrate. The medium contained (g./l. tap water): cane molasses, 280 (equivalent to 157 g. sucrose); KH_2PO_4 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; NH_4NO_3 , 2.5; adjusted to pH 5.0. This medium was dispensed in 100 ml. amounts in 500 ml. conical flasks and autoclaved at 116° (10 lb./sq.in. pressure) for 15 min. The inoculum consisted of 3×10^6 spores harvested from beer wort agar slopes and suspended in water containing Soaxit (sodium dioctyl sulphosuccinate, 1/10,000); incubation was at 28° for 5–9 days. Cultures were analysed after different periods since information on rate, as well as degree, of citric acid yields was desired.

RESULTS

Table 3 shows the yields of citric acid obtained by surface fermentation of cane molasses, and brings out the interesting correlation between the fermentative activity of the various strains and their acid unitage. The mutant strains gave uniformly higher yields of citric acid (confirmed by chromatographic analysis) than the parent strain; the increase was approximately sixfold with the most active fermenters (cf. 72-4 and V7-1). Further, it will be noted that successive mutations (see Gardner *et al.* 1956) resulted in progressively increasing yields

as, for instance, in the series X20, V7, V7-1 and V7-2. These consistently high yields (greater than 30 % for five out of the seven mutants) were most encouraging in view of the high metal content of cane molasses. In this connexion it might be noted that the calculated manganese content of our medium was about 6 mg./l.—6000 times greater than the optimal concentration found by Shu & Johnson (1947) for strain 72-4. Further, the stability of strains X20 and V7-1 was evident in that they showed no decrease in acid production after fifteen successive subcultures during a period of 4 months on beer wort agar.

Table 3. *Correlation of 'acid unitage' with yields of citric acid in surface fermentation of cane molasses*

Strain	Paper culture after 5 days			Yield of citric acid* (%)
	Colony diameter (mm.)	Acid zone diameter (mm.)	Acid unitage	
72-4	15	25	1.7	5
X20	6	14	2.3	14
V4	4	10	2.5	20
V4-1	2	11	5.5	32
V4-2	3	17	5.7	32
V7	4	21	5.2	30
V7-1	4	22	5.5	32
V7-2	3	20	6.6	35

* Calculated on total sugar available.

The second point worth mentioning is that our new method of paper culture has proved eminently suitable for isolating and selecting high acid-yielding mutants. That direct selection of mutants on their acid unitage value is reliable has been confirmed by surface (Table 3) and submerged fermentations (Gardner *et al.* 1956). A good citric acid-producing strain should have an acid unitage of 5 or more.

DISCUSSION

The main object of this paper has been to draw attention to the technique of cultivating mould spores on absorbent paper previously soaked in an indicator medium, as a method of isolating and characterizing biochemically interesting mutants. A specific application of this method is described here for selecting high yielding citric acid-producing mutants of *Aspergillus niger*.

Several advantages over the usual agar-containing media may be claimed for paper culture. For example, colonies of *Aspergillus niger* grow compactly on paper culture with diameters less than 2 cm. after 4 days, and the diffusion of acid metabolic products is similarly restricted. Thus it is possible to cultivate on the same surface area many more colonies than would be accommodated on the agar media. In this way paper culture greatly facilitates the isolation and characterization of mould colonies with economy of space, media and equipment. Further, in the present instance it was possible to estimate the potentiality of isolates directly by a ratio termed the acid unitage, obtained by dividing the diameter of the acid zone by that of the colony. A strain having



Fig. 1

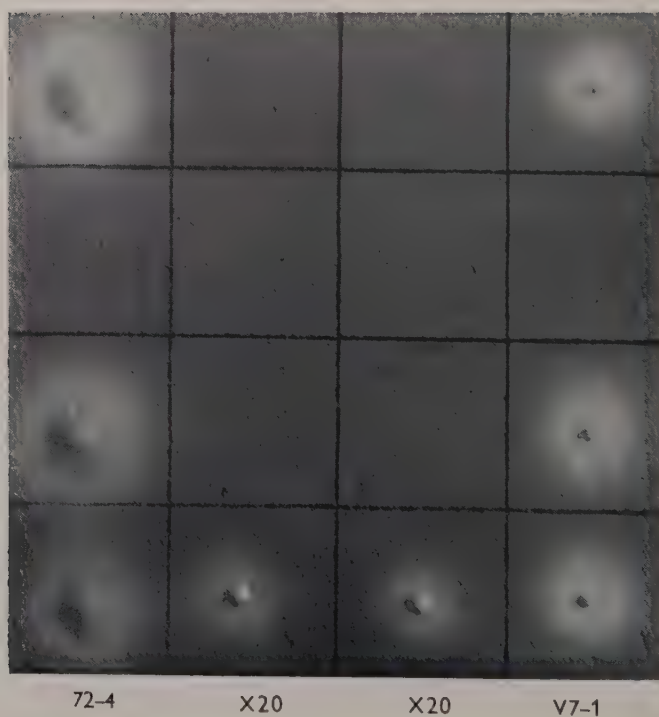


Fig. 2

L. V. JAMES, S. D. RUBBO & J. F. GARDNER—ISOLATION OF MUTANTS
BY PAPER CULTURE. PLATE 1

(Facing p. 227)

an acid unitage greater than 5 could be classed as a high citric acid-producer and worthy of further study. While our interest has been directed towards selecting mutants producing high yields of citric acid the method could be easily modified for other purposes, e.g. large-scale testing of antifungal agents or the selection of auxotrophic mutants of fungi.

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EXPLANATION OF PLATE

- Fig. 1. Ten-inch Petri dish containing sporulating parent strain and non-sporulating mutants on paper culture after 6 days of incubation at 28°.
- Fig. 2. Colonies of *Aspergillus niger* strains 72-4, X20 and V7-1 on paper culture, showing acid zones after 4 days of incubation at 28°. Note the varying size of the colonies and acid zones. For calculation of degree of acid production, 'acid unitage', see Table 3.

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Production of Citric Acid by Mutants of *Aspergillus niger*

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SUMMARY: Mutants of *Aspergillus niger*, Wisconsin strain 72-4, were produced by multiple X-ray and ultraviolet irradiation. The mutants differed from the parent strain culturally and in citric acid production. Comparison between the yields of citric acid from the parent strain and mutants showed significant increases with the latter. In crude sugar media, such as ferrocyanide-treated brown sugar, yields of citric acid equivalent to 80 % of the sugar fermented were obtained in aerated culture, whereas only 21 % was obtained with the parent strain.

The factors which govern citric acid production by *Aspergillus niger* in submerged culture, particularly the influence of the metal constituents of the medium, were investigated in detail by Shu & Johnson (1947, 1948*a, b*), and their findings were confirmed and extended by Tomlinson, Campbell & Trussell (1950, 1951). The small amounts of iron, zinc, copper and manganese which are essential for optimal growth (Steinberg, 1935) exceed the concentrations which favour citric acid production. Thus one step towards providing suitable conditions for acid accumulation is to decrease these metal concentrations well below those required for optimal growth. Even so, it is extremely difficult to obtain constantly the highest yields of citric acid, especially from crude cane sugar, by the submerged culture method.

Three approaches to this problem are possible: (1) to strip the medium of its excess metal contaminants by ion-exchange techniques, as described in a patent issued to Miles Laboratories (1952), or by potassium ferrocyanide precipitation (Gerhardt, Dorrell & Baldwin, 1946; B.I.O.S. Final Report 220, 1946; Clement, 1952); (2) to add a growth-inhibitory agent such as methanol which was found to increase the metal concentrations permissible (Moyer, 1953); (3) to isolate metal-tolerant mutants.

In regard to this third possibility Perlman (1947), who investigated some seventy-five strains of *Aspergillus niger* for ability to produce citric acid in submerged culture, found considerable variation among them and reported that some were less affected by metal ions than others. Quilico, Panizzi & Visconti (1949) obtained mutants by X-ray irradiation which gave increased yields on Currie's defined medium (1917); they did not report on the yields of citric acid obtained from crude sugar substrates. Yuill (1951) commented briefly on citric acid production by mixed cultures of *A. niger*, some of them mutants. He stated that when two unrelated high acid-producing strains were grown together, the yield was lower than that given by either separately, but no such decrease was observed when the strains were related as mutant and parent, or as mutants from the same parent; no information was given about the ability of these mutants to produce acid from crude sugars more efficiently than the parent strain.

The present work is concerned with the study of citric acid production by mutants of *Aspergillus niger* in metal-contaminated crude cane sugar by submerged (shaken or aerated and stirred) culture. A method developed for selection of such mutants is described separately (James, Rubbo & Gardner, 1956).

METHODS

Organism

The parent culture was *Aspergillus niger* Wisconsin strain 72-4 (Perlman, Kita & Peterson, 1946). For irradiation and fermentation experiments, cultures were grown on the 14 % sucrose agar defined medium described by Shu & Johnson (1947) and on beer wort agar. On these media sporulation was prolific after 3-5 days of incubation at 28°.

Irradiation techniques

X-ray irradiation. Freshly formed spores were spread aseptically on the surface of a 4 % agar gel and exposed to X-rays in doses of 45,000 and 60,000 r. The spores were then washed off the agar with water containing a wetting agent, Soaxit (sodium dioctyl sulphosuccinate, 1/10,000), and the suspension filtered through sterile cotton-wool to remove clumps. The death-rate was estimated by comparison of the viable count (plate method) with a haemocytometer count of the total number of spores, and found to be 99 % with a dose of 45,000 r. and 99.9 % with 60,000 r. In later experiments 1 ml. of an aqueous suspension of spores, previously filtered through cotton-wool and containing 2×10^6 spores, was irradiated in a small Petri dish covered with cellophan.

Ultraviolet irradiation. Filtered suspensions of spores in water, containing 2×10^6 spores/ml., were irradiated, at room temperature (*c.* 20°) in a clear quartz tube of 2 cm. diameter, by a vertical 15-watt G.E. low-pressure germicidal lamp (2537 Å.). The tube was rotated at 180 r.p.m. with its axis parallel to and 12 cm. from the lamp. The irradiations were performed in a dark room and the irradiated suspensions were shielded from light until plated out in order to minimize any photoreactivation effects. Exposure for 15 min. gave a 99.9 % kill.

The plan for preparation of high-yielding mutants was similar to that outlined by Dulaney, Ruger & Hlavac (1949) for improving streptomycin yields by *Streptomyces griseus*, in that it involved exposure of the spores of successively induced mutants to mutagenic agents. All strains selected were preserved by lyophilization of spores harvested from beer wort agar.

Fermentation techniques

Submerged culture methods. Some mutants which showed superior acid-producing ability on cane molasses substrate by surface culture were tested as submerged cultures in flasks on a rotary shaker (1 in. throw, 200 r.p.m.) at 28° and finally in 5 l. fermentation flasks equipped for aeration and agitation.

The shaken flasks, of 500 ml. capacity and loosely plugged with cotton-wool, contained 50 ml. of medium. Three preparations containing sucrose were used: white sugar of the ordinary commercial grade; a brown crystalline product ('brown sugar'); cane molasses. Their content of those trace metals important in the fermentation is shown in Table 1.

Table 1. *Metal content in different batches of cane sugar*

Type of sucrose preparation	Metal content (p.p.m.)			
	Copper	Iron	Zinc	Manganese
White sugar	0.06-0.20	1.42-3.00	0.24-1.00	0.45-1.40
Brown sugar	3.3-4.7	65-88	1.1-5.7	1.6-7.0
Molasses	7.6-7.8	104-360	4.4-57	39-49

All media contained (g./l.): sucrose preparation, 140; KH_2PO_4 , 1.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; NH_4NO_3 , 2.5. In the case of the white sugar medium, trace metals were added as follows (mg./l.): $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.2; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0; these being the quantities given by Shu & Johnson (1947). The pH value was adjusted to 3.0 for white sugar, 5.6 for brown and 5.0 for molasses. The flasks were inoculated with a suspension of mycelial fragments prepared in shaken cultures containing plastic beads. Cultures were incubated for 9 days before being analysed for acidity and residual sugar.

For aerated submerged fermentations a battery of four 5 l. round-bottom reaction flasks of Hysil glass mounted in a water-bath at 28° was used; the equipment was similar to that described by Kelly, Miller & Hale (1952). Air inlet tubes and baffle plates were glass, stirrers were of glass or stainless steel. The rate of air flow was 4 l./min. and the speed of the stirrer was 600 r.p.m., both being the maximum obtainable with our equipment.

Fermentations were started by inoculating 1 l. of 2%, w/v, sugar medium with 100 ml. of a suspension of mycelial fragments. Incubation with aeration for 24 hr. produced an actively growing mycelial mass, to which 3 l. medium containing high sugar and low ammonium nitrate (187 g. and 0.83 g./l. respectively) were added. The final concentration of sugar in the combined media was 14%.

When potassium ferrocyanide was used in the brown sugar medium it was added to the hot medium after autoclaving, usually 0.25-0.50 g./l. for the shaken cultures and 0.75-0.90 g./l. for aerated submerged fermentation. In cane molasses a much higher concentration (3.0 g./l.) was required for high yields in shaken cultures.

Acidity was determined by titration with 0.25 N-NaOH and residual sugar by Cole's ferrieyanide method (1933). At the conclusion of a fermentation the acid products were identified and their proportions estimated by partition chromatography, with butanol + water containing 2N-formic acid as solvent (Lugg & Overell, 1948).

RESULTS

Origin and description of mutant strains

The distinguishing cultural characteristics of the strains of *Aspergillus niger* investigated are shown in Table 2 and Pl. 1. It will be seen that the mutants are easily distinguished by their restricted colony development and delayed sporulation. After 3 days of incubation on Czapek-Dox medium the mutants gave raised non-sporulating compact colonies (popcorn colonies), while the parent strain 72-4 gave a flat spreading colony with spores. When grown on beer wort agar slopes strain 72-4 spored prolifically in 2-3 days whereas, after 7 days, the felt of mutant X 20 was unevenly covered with spores. It is interesting to note that mutant V7-1 spored freely only on the upper portion of the slope after 9 days of incubation (Pl. 1), a character shared by all the other mutants except X 20.

Table 2. *Origin and distinguishing features of Aspergillus niger strains*

<i>Aspergillus niger</i> strain	Origin	Time and degree of sporulation on beer wort agar (days/degree*)	Colony size on Czapek-Dox agar after 8 days (central point inoculation) (diameter, mm.)
72-4 (Wild type)	University of Wisconsin	3/ + + + +	35
X 20 (mutant)	X-ray irradiation of 72-4	7/ + + +	10
V 4 (mutant)	UV irradiation of X 20	9/ + +	8
V 4-1 (mutant)	UV irradiation of V 4	14/ +	8
V 4-2 (mutant)	UV irradiation of V 4-1	14/ +	8
V 7 (mutant)	UV irradiation of X 20	9/ + + +	8
V 7-1 (mutant)	UV irradiation of V 7	9/ + +	8
V 7-2 (mutant)	UV irradiation of V 7-1	9/ + +	8

* + + + + = entire felt covered with black spores; + + + = 3/4 felt covered with black spores; + + = 1/3 felt covered with black spores; + = < 1/3 felt covered with black spores.

When grown on the surface of liquid media the mutants in general developed felts which were lighter and more friable than those of the parent strain 72-4. On cane molasses mutant V7-1 grew as a light, non-sporing felt covering the entire surface of the medium, mutant X 20 grew as an incomplete moderately sporing felt, while the parent strain 72-4 produced a tough, heavily sporing band of felt restricted to the edge of the medium. Submerged cultures in shaken flasks reflected the restricted mycelial development of the mutants which was seen on solid media. These characteristics, like the fermentative ability, have remained unchanged through many subcultures over the past 2 years.

Fermentative activity in submerged culture

Fermentations in shaken flask culture with parent strain 72-4 and mutants X 20 and V7-1, using various sugar substrates were studied. The results are summarized in Fig. 1. The average yield from all substrates was higher with the mutants than with the parent culture. With crude sugars the superiority

of the new strains was most evident. On treated and untreated molasses the yields of acid with mutant V7-1 were about twice those obtained with mutant X20, a similar ratio having been obtained in surface fermentation (James *et al.* 1956).

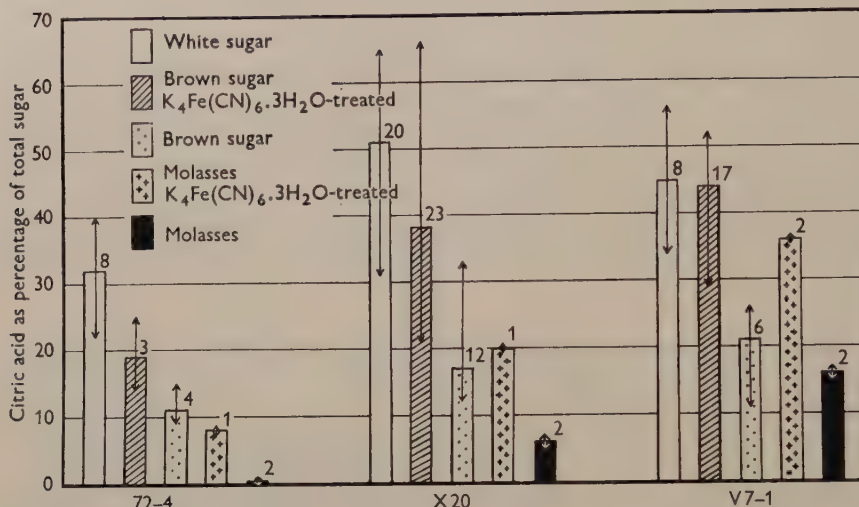


Fig. 1. Average yields of citric acid (columns) and range of variation (arrows with number of fermentations analysed) in 9-day shaken flask cultures at 28°.

The results recorded in Table 3, based on those shown in Fig. 1, indicate that the yields of acid progressively decreased as the metal content of the medium (contributed by the sugar) increased. The loss of efficiency was most pronounced with the parent strain and least with mutant V7-1. However,

Table 3. *Influence of substrate on yields of citric acid*

Nine-day shaken flask fermentation at 28°.

Strain	Yields of citric acid*				
	White sugar	Brown sugar	Brown sugar	Molasses	Molasses
	(%)	$K_4Fe(CN)_6 \cdot 3H_2O$ -treated	untreated	$K_4Fe(CN)_6 \cdot 3H_2O$ -treated	untreated
72-4	100	61	33	23	0
X20	100	74	32	34	11
V7-1	100	98	52	71	32

* For purposes of comparison the yields of citric acid from white sugar are calculated as 100% and the yields from other sugars adjusted accordingly.

from an industrial point of view, it is apparent that untreated cane molasses is unsuitable as a substrate for citric acid production with the mutants available, although mutant V7-1 gave outstandingly good yields in ferrocyanide-treated sugar or cane molasses.

In the fermenters, where aeration was combined with mechanical agitation, the results (Fig. 2) generally confirmed the findings with surface and shaken fermentations, mutant V7-1 being superior to mutant X20 and this in turn superior to parent strain 72-4. It is worth noting that mutant V7-1 in ferrocyanide-treated brown sugar was better in aerated fermenters than in shaken flasks, whereas with mutant X20 the difference was negligible.

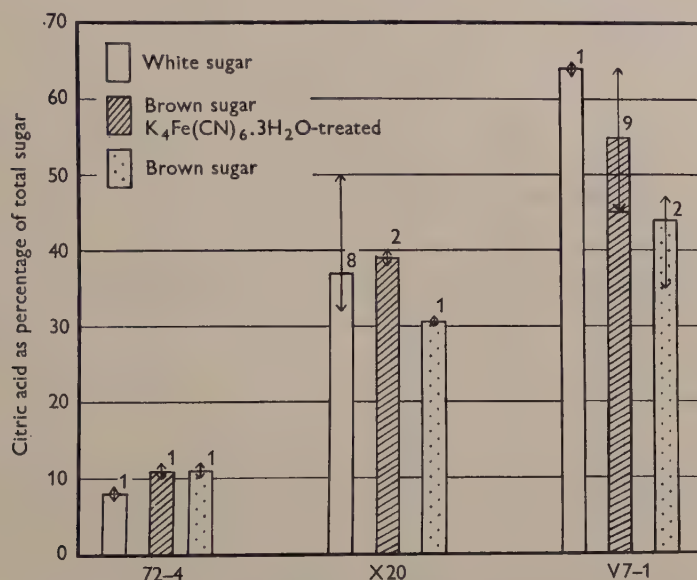


Fig. 2. Average yields of citric acid (columns) and range of variation (arrows with number of fermentations analysed) in 11-day aerated and stirred cultures at 28°.

A closer analysis of the aerated fermentation is given (Fig. 3) for brown sugar media, with and without ferrocyanide treatment. It will be seen that the production of citric acid was most rapid with mutant V7-1, reaching a yield of 64 % in 9-11 days in the ferrocyanide-treated medium. At the conclusion of these fermentations, when the acidity had reached its maximum, the culture fluid usually contained 1-2 % sugar. Therefore, when the results are calculated on sugar consumed the yields are higher than those shown in Figs. 2 and 3, reaching 21 % for the parent strain 72-4, 48 % for mutant X20 and 80 % for mutant V7-1 in 9 days in the best runs. Such yields compare favourably with those regarded as satisfactory in an industrial process.

Utilization of citric acid as a substrate

It was thought that the difference in citric acid production between the parent strain and mutants of *Aspergillus niger* might be due to differences in their ability to utilize citric acid as a substrate, the parent being more active than the mutants. In order to test this point surface felts and submerged pellets of *A. niger* mycelium were harvested from sucrose medium, washed and

transferred to a medium containing 6% citric acid as sole source of carbon. In addition, parallel tests were done in 6% citric acid medium containing 4% sucrose. These replacement cultures were incubated at 28° in static and shaken flasks and the utilization of citric acid determined by titration with 0.25N-NaOH and also colorimetrically (Saffran & Denstedt, 1948). The results

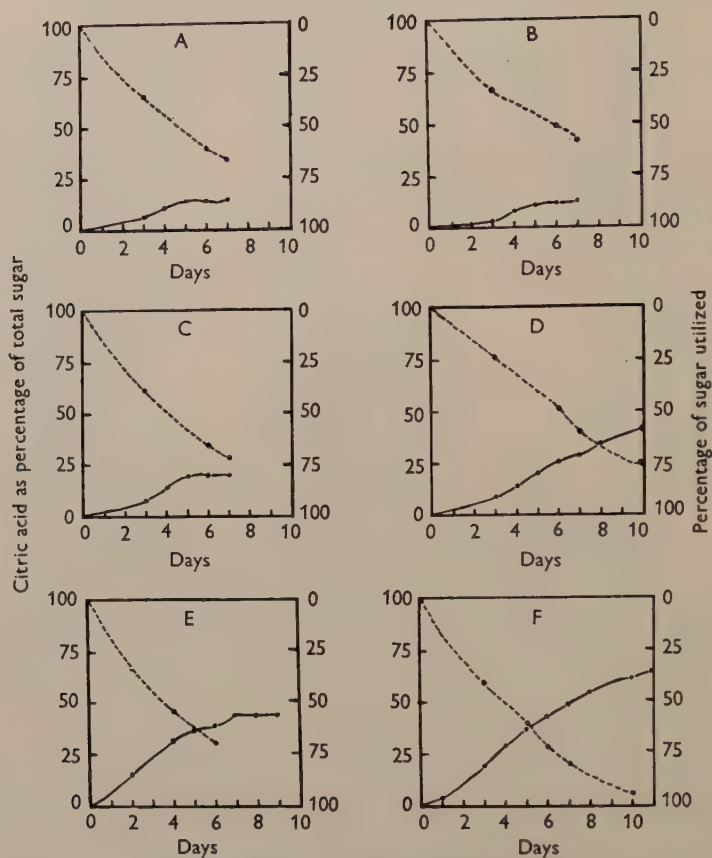


Fig. 3. Rate of citric acid production (—) and sugar utilization (---) in aerated and stirred cultures at 28°.

A, B, parent strain 72-4 on untreated and on $K_4Fe(CN)_6 \cdot 3H_2O$ -treated brown sugar.

C, D, mutant X20 on untreated and on $K_4Fe(CN)_6 \cdot 3H_2O$ -treated brown sugar.

E, F, mutant V7-1 on untreated and on $K_4Fe(CN)_6 \cdot 3H_2O$ -treated brown sugar.

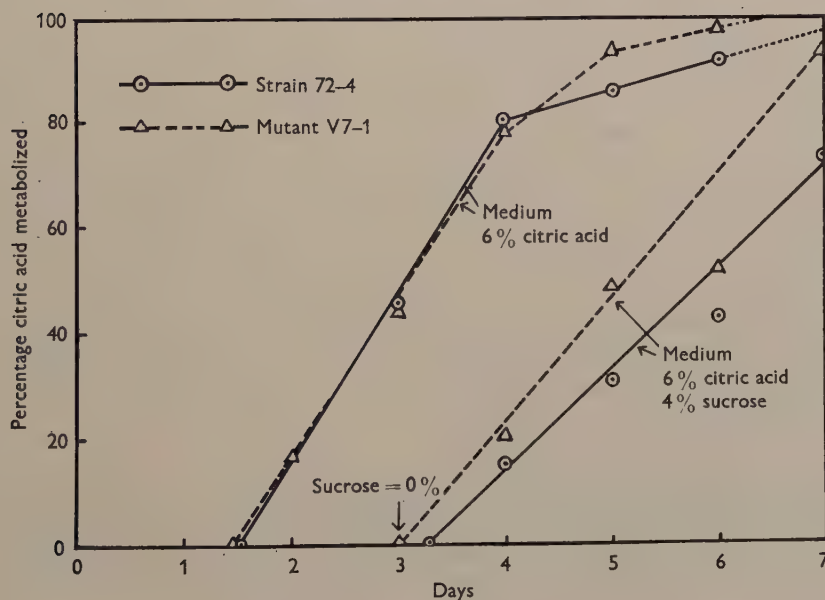
(Table 4) showed that the rate of disappearance of citric acid in both static and shaken cultures was similar for the parent strain and mutants. The discrepancy between the total titratable acid and citric acid estimations, which developed towards the end of the fermentation, indicated that acids other than citric were being produced. When sucrose was present (Fig. 4) there was no apparent utilization of the citric acid by any of the cultures until the sugar concentration had fallen to zero. These findings clearly showed that the high yields of citric acid in the mutant cultures (and their restricted growth rate)

were not due to their inability to metabolize the citrate formed. In any case it appears unlikely that any of the cultures will attack citrate while sucrose is present in the medium. It is obviously important to take advantage of the citrate-sparing effect of sucrose if highest yields of citric acid are to be obtained commercially.

Table 4. Utilization of citric acid by strains of *Aspergillus niger*

Strain	Method of cultivation	Citric acid yield in culture fluid				
		Initial		After 4 days; by titration	After 7 days	
		Titration	Colorimetric		By titration	Colorimetrically
		Citric acid (mg./ml.)				
Parent 72-4	Surface	59	59	41	13	2
	Shaken	63	68	12	5	N.D.
Mutant X20	Surface	57	59	26	9	0
	Shaken	65	70	8.5	3.5	N.D.
Mutant V7-1	Surface	58	59	25	7	0
	Shaken	66	N.D.	13.5	2	N.D.

N.D. = not determined.

Fig. 4. Utilization of citric acid by strains of *Aspergillus niger*.

DISCUSSION

The aim of this investigation was to study the fermentative activity of mutants of *Aspergillus niger*. These mutants were induced by X-ray and ultraviolet irradiation of spores and selected by paper culture technique

(James *et al.* 1956). They were culturally distinct from the parent strain and stable in all characteristics studied.

The results show that all the mutants were markedly more active in producing citric acid than the parent strain under all conditions of fermentation, namely in static, shaken and aerated submerged cultures with commercially pure sucrose or crude sucrose such as cane molasses. In aerated fermentations these differences were most pronounced in that one mutant, V7-1, gave fourfold to sixfold higher yields of citric acid than the parent strain 72-4. Mutant V7-1 appears to be suitable for large-scale production of citric acid since it is capable of giving yields of citric acid equivalent to 80 % of the sugar fermented in ferrocyanide-treated crude brown sugar media. With beet molasses, a substrate similar in metal content to brown cane sugar, comparable results might confidently be expected. Unfortunately, beet molasses was not available to us in the present study.

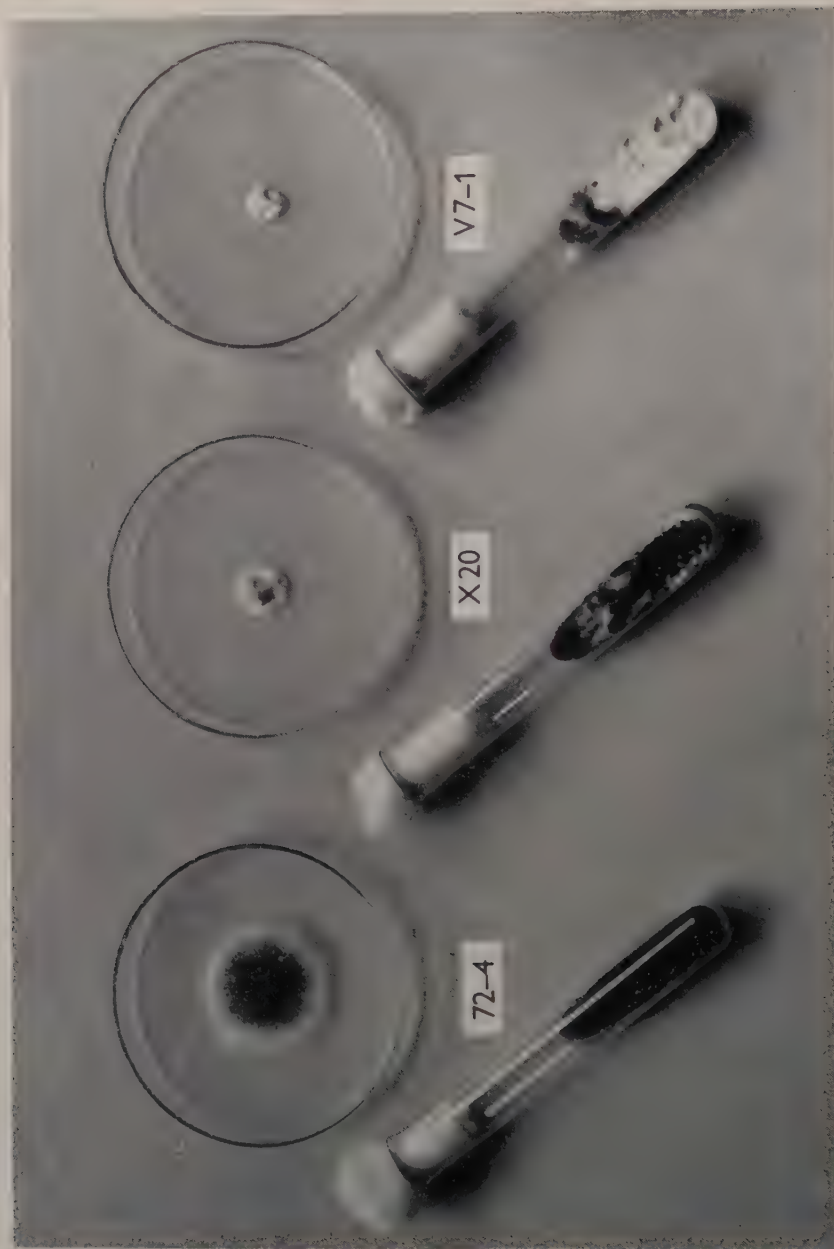
Several points of interest emerge from this work. In the first place it was apparent that the best strains are multiple-step mutants produced by exposure to mutagenic agents. These differed from the parent strain in requiring up to 9 days for sporulation, in growing as small compact colonies (Table 2) and in producing lighter and more friable felts on liquid media as well as smaller amounts of mycelium in submerged culture. The constant correlation between high yields of citric acid and restricted development suggests that the mutants may be deficient in certain metal-dependent enzymes associated with growth, in particular those concerned with the utilization of citrate. Experiments reported here (Table 4, Fig. 4), indicate, however, that the restriction of growth and the enhanced production of citric acid were not concerned with the inability of the mutants to utilize citric acid as a substrate.

The yields quoted for *Aspergillus niger* 72-4 on white sugar medium are considerably lower than those reported by Shu & Johnson (1947, 1948*a, b*); this is probably due to a higher concentration of metals in our media. For example, the concentration of manganese (60-196 $\mu\text{g./l.}$) in the white sugar medium was considerably in excess of the optimal level of 1 $\mu\text{g./l.}$ determined by Shu & Johnson. Little attention was given to the purification of media in this work since our aim was to produce organisms capable of giving high yields of citric acid from impure substrates.

It is with pleasure that the authors thank Kraft Foods Ltd. (Melbourne) for initiating this investigation and providing financial assistance for one of us (J. F. G.). Our thanks are also due to Dr K. T. H. Farrer of the same organization for helpful advice, to Miss A. Macauley for technical assistance and to Prof. W. H. Peterson for supplying the parent culture of *Aspergillus niger* strain 72-4.

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BY *A. NIGER* MUTANTS. PLATE 1

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EXPLANATION OF PLATE

Comparison of colonial characteristics (6 days, 28°, Czapek-Dox plates) and sporulation (9 days, 28°, on beer wort agar slopes) of the parent strain 72-4 and mutants X20 and V7-1.

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Nitrite Production by Heterotrophic Bacteria

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SUMMARY: Heterotrophic bacteria were studied which produced nitrite in the presence of ammonia and in the absence of nitrate. A soil extract medium was prepared which allowed good growth as well as nitrite production in the absence of nitrate. Quantitative data are recorded showing that ammonia decreases as nitrite accumulates when four different cultures are grown in the soil medium. Resting cell studies add further evidence that some heterotrophic bacteria can convert ammonia to nitrite.

A defined medium was prepared containing glucose or sodium acetate as the carbon source and NH_4Cl as the nitrogen source. This medium supported growth and nitrite production; however, optimum conditions for growth were not established. Neither growth nor nitrite accumulation was as great in defined media as in soil-extract media. Results from defined media and from resting-cell studies rule out the possibility of any nitrate contamination.

Early in the literature reports were published by Fremlin (1903, 1914, 1929), Makrinoff (1909), Cutler (1930), Cutler & Mukerji (1931), Cutler & Crump (1933), Crump (1935), Nechaeva (1947) and others suggesting the possibility of nitrification by heterotrophic bacteria. Recently Fisher, Fisher & Appleman (1952) and Hutton & ZoBell (1953) have added more data supporting these early observations. Isenberg *et al.* (1954) described a streptomycete which produces nitrite from urethane.

This paper presents evidence that certain Gram-negative bacteria isolated from soil form nitrite in the presence of ammonia in defined and undefined media. Ammonia disappears as nitrite is produced. Special precautions have been taken to exclude nitrate from the media.

METHODS

Bacteria. Sixteen nitrite-producing cultures were obtained from soil by enrichment techniques. These were shown to be pure. The organisms were found to be Gram-negative short rods probably belonging to group 2 or 3 of Taylor & Lochhead's (1938) classification of soil bacteria. This classification, based on morphology and Gram-staining reactions of cultures grown on soil extract semi-solid agar, has eight large subdivisions, group 2 consisting of short Gram-negative rods and group 3 composed of short Gram-variable rods.

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Organisms in group 2 were least active physiologically and were thought to comprise much of the indigenous soil flora. Organisms selected for this particular study produced nitrite in the presence of ammonia and in the absence of nitrate. Cultures designated 8₅, 14₉ and 54 did not produce acid when grown in the presence of glucose, mannitol, lactose, sucrose or maltose. They were H₂S- and indole-negative, non-motile, produced an alkaline reaction or reduction and peptonization in litmus milk, and they were able to reduce nitrate. Cultures 19, 20, 31, 33, 40, 43 and 45 did not produce acid in the presence of the sugars mentioned. They were H₂S- and indole-negative and did not reduce nitrate. They produced an alkaline reaction in litmus milk, and were motile. Culture 13 was even less active physiologically in that acid was not produced in the sugar broths; there was no growth in H₂S, indole, litmus milk or gelatin media and it was found to be non-motile. Cultures 10, 15 and 17 produced acid and gas in media containing the five sugars; H₂S and indole were not produced; they could reduce nitrate, formed acid and coagulated litmus milk, liquefied gelatin, and were motile. Cultures 30 and 34 produced acid in glucose but not in other sugars. Tests were negative for H₂S and indole production, for gelatin liquefaction, and for motility. They could reduce nitrate but showed no change in milk. Further attempts to classify these organisms, including a key for the separation of isolates, were presented by Fisher (1953). All cultures grew on nutrient agar but would not grow on Winogradsky's autotrophic media for nitrifiers. Cultures 8₅, 10, 14₉ and 54 were selected for intensive study since these organisms showed consistently heavier cell growth in most of the culture preparations.

Media. The undefined medium generally employed contained 50 % (v/v) soil extract (Lochhead & Thexton, 1952), 20 % (v/v) phosphate buffer, pH 7·4, and 0·01 % (w/v) (NH₄)₂CO₃. The components and preparation of this medium will be described. Soil extract: 1 kg. of soil from the Los Angeles area (very low or negative for nitrite and nitrate) was added to 1 l. distilled water and the mixture autoclaved at 15 lb./sq.in. for 30 min. A small amount of CaCO₃ was introduced into the suspension after autoclaving while the material was still hot. The preparation was filtered. This was considered 100 % soil extract. Buffer: best results were obtained when phosphate buffer, pH 7·4, was prepared as follows: Na₂HPO₄·7H₂O 2·745 g. dissolved in 200 ml. H₂O; KH₂PO₄ 0·4213 g. dissolved in 200 ml. H₂O. The two solutions were mixed in equal volumes. The 50 % soil extract medium was assembled in the following proportions:

Soil extract	50 ml.
Mixed phosphate buffer	20 ml.
Distilled water	30 ml.
(NH ₄) ₂ CO ₃	0·01 g.

The medium was autoclaved at 15 lb./sq.in. for 20 min. which allowed the pH value to hold between 7·2 and 7·5 under the above conditions. A 10 % soil extract medium had the same constituents as the 50 % medium except that 10 ml. of the concentrated soil extract was used instead of 50 ml., maintaining

the total volume at 100 ml. When a solid medium was required, 1.25 % washed agar was added to the above medium. Stock cultures were stored in 0.3 % semi-solid soil extract agar. Soil extract media were placed in 6 oz. bottles so that the bottles were approximately half full or in screw cap test-tubes (16 × 120 mm.) and half-filled before autoclaving. After inoculation, tube or bottle tops were generally screwed on lightly so that anaerobiosis was not obtained.

All glassware used was acid cleaned to eliminate traces of nitrate. Care was taken to maintain nitrate-free water and reagents. Media controls were run with each experiment to be sure the media did not pick up any nitrogenous oxides from the air which might give positive nitrite tests.

The defined medium contained 0.5 % glucose or 0.5 % sodium acetate as the carbon source. Ammonium chloride was used in concentrations of 0.01 or 0.03 %. The other constituents consisted of 0.61 % Tris (hydroxymethyl) aminomethane buffer adjusted to pH 7.4 with HCl, 0.01 % NaHCO_3 , 0.06 % MgSO_4 , a trace of CaCl_2 and phosphates. The medium was autoclaved at 15 lb./sq.in. for 20 min.

Analytical procedures. A modified Griess-Illosva method (Griess, 1858; Illosva, 1889) was used for nitrite determinations. Reagents were prepared according to the procedure in *Methods of Analysis of the Association of Official Agricultural Chemists* (1935). By this method 25 ml. of the diluted sample were tested. Two drops of concentrated HCl were added to the sample, followed by 1 ml. of sulphanilic acid solution and the contents mixed. 1 ml. of α -naphthylamine hydrochloride reagent was then introduced, the tube contents were mixed thoroughly and readings were taken in 30 min. Duplicate portions of each of several dilutions were tested as described. A standard curve was prepared, but each time determinations were run several dilutions of the standard samples were tested to be sure the curve was still accurate. Transmission readings were made in the Klett-Summerson colorimeter (540 filter) or the Beckman DU spectrophotometer (525 m μ . wavelength). Wallace & Neave (1927) reported that the colorimetric nitrite test used was sensitive to 1 part of nitrite in 100 million of solution.

A modification of Bray's method (1945) to determine nitrate was used. This follows the same procedure as the nitrite test except that an acid suspension of powdered zinc was added to a mixture of the reagents and these in turn were introduced into the sample to be tested. A red colour developed just as in the nitrite test so that the same standard curve was used for both determinations. Known quantities of nitrate were also tested in duplicate as controls, each time to insure the sensitivity of the method and reliability of reagents. This method for nitrate was found to be as sensitive as the nitrite test.

Qualitative tests for nitrite and nitrate were measured using the reagents described for quantitative testing, except that values obtained were designated 1, 2, 3 and 4 plus depending on relative colour intensities upon gross examination.

Ammonia was determined by the colorimetric nesslerization method as listed in Wilson & Knight (1949). A distillation method was used to obtain

samples for colorimetric analysis. An ammonia-free water blank was boiled 10 min. to remove any ammonia from the apparatus. A 15 ml. sample of culture medium centrifuged free from organisms was placed in the ammonia-free flask containing 10 ml. distilled water. 10 ml. of concentrated NaOH was added to the closed system and by heating gently for 10 min. ammonia was driven over through a water-cooled condenser to a flask containing a weak solution of HCl. The total volume of distillate collected was recorded since portions of this distillate were used to determine the amount of ammonia/ml. in the original sample. Two to three samples of the same culture medium were distilled over as described in the method above. Triplicate samples of each distillate were tested colorimetrically. The values obtained corresponded very closely.

To test qualitatively for the presence of ammonia, Nessler's reagent was added to a sample solution and the development of a dark yellow colour was considered a 4 plus positive test. Gradations less than this in colour intensity were designated 3, 2 and 1 plus positive.

RESULTS

Optimum conditions for growth and nitrite production. A 10 % soil extract medium had been used to isolate the cultures studied. Since this medium was found to be minimal for growth a 50 % soil extract medium was prepared and adjusted to various pH values with HCl or NaOH before autoclaving. The medium was positive for ammonia and negative for nitrite and nitrate as determined by procedures described in the methods section. The pH value of the medium was adjusted to the values: 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0 and 8.4. Of the four cultures tested in these media (8₅, 10, 14₉ and 54) growth of all cultures occurred from pH 6.8 to 8.0. Growth was best at pH 7.4 and good at pH 7.2. Nitrite production was good at pH 7.4 and 7.2.

It was shown that nitrite production increased with an increase in organism numbers. Observations indicated that maximum growth in 50 % soil extract media was obtained in about 2 days while nitrite formation continued to increase until a peak was reached in approximately 4 days when the cultures were incubated at 30°. The fact that nitrite production increases with an increase in number of organisms and continues to increase for a short time after maximum growth is attained, suggests that nitrite accumulation is due to the biological agent.

Nitrite accumulation and ammonia disappearance in soil extract medium

The four cultures were grown in 50 % soil extract medium at 30° in 6 oz. screw-cap bottles half-filled with medium. The cultures were incubated for 22 days. The medium, centrifuged free of cells, was then tested for nitrite, nitrate and ammonia by the quantitative methods described. The uninoculated control media contained only trace amounts or no nitrite or nitrate so that these results are recorded as 'less than' a certain value. Nitrite and nitrate

ions have never been found to be present in the soil extract media used in more than trace amounts.

Values for ammonia nitrite and nitrate, as well as the possible percentage conversion of ammonia nitrogen to nitrite nitrogen, are recorded in Table 1. These data show that ammonia disappears as nitrite accumulates when cultures 8₅, 10, 14₉ and 54 grow in soil extract medium. Eighty-seven % of the ammonia lost was recovered as nitrite in culture 54. This was the highest value obtained. Sixty-three % of the ammonia lost was recovered as nitrite in culture 14₉. This was the lowest recovery value observed. These data show that nitrite accumulated as ammonia simultaneously disappeared in the absence of nitrate, suggesting a conversion of ammonia to nitrite.

When preparations of 100 % soil extract were tested by micro-Kjeldahl determinations, a different value of total nitrogen was obtained for different soil samples tested. This was expected, due to the many variables in soil as well as due to evaporation and concentration which occurs during the autoclaving process. A defined medium for nitrification studies was considered necessary to eliminate such variables and rule out any suspicion of conversion of nitrate to nitrite. This is considered later.

Increase in nitrite production with an increase in ammonia concentration. In order to show that nitrite accumulation increases with increased amounts of ammonia, 50 % soil extract buffered at pH 7.4 with Tris was used as one medium, while other portions were fortified with 0.01 or 0.03 % NH_4Cl . Cultures were incubated 10 days at 30°, centrifuged free of cells and tested for nitrite and nitrate. These findings are recorded in Table 2. Nitrite production was greatly increased when 0.01 % NH_4Cl was included in the medium. When the NH_4Cl concentration was increased above the 0.01 % level no rise in the nitrite concentration was obtained. The optimum concentration for nitrite production by these cells in these media seemed to be at or near the 0.01 % NH_4Cl concentration when 50 % soil extract is present. Soil extract alone gave a 4 plus positive qualitative Nessler's test for ammonia, but this concentration apparently is not high enough to support optimum heterotrophic nitrite production. Tests for nitrate were negative on all solutions used.

Growth and nitrite production in defined media. Organisms designated 8₅ and 14₉ were inoculated into the two defined media, one containing sodium acetate as the carbon source and the other containing glucose. Nitrite production under these conditions is reported in Table 3. In both cases the growth obtained in defined media was somewhat less than maximum compared to that observed when these bacteria were grown in soil extract media.

When defined media were used for growth and nitrite formation, nitrite increased as the cultures were incubated for 7 days. After 7 days of growth, nitrite values reached a peak, remained stationary for several days then dropped slowly. Values reported for nitrite production by heterotrophic bacteria in defined media are lower than those in soil extract media. However, since the sensitivity of the nitrite and nitrate methods permit the measurement of values much lower than those obtained and since the media are defined and all possibility of nitrate inclusion or contamination is eliminated,

Table 1. *Ammonia nitrite and nitrate determinations showing the possible percentage conversion values of ammonia nitrogen to nitrite nitrogen by four heterotrophic bacteria*

Medium: 50 % soil extract containing 0.01 % $(\text{NH}_4)_2\text{CO}_3$ and 6.6×10^{-3} M-phosphate buffer, pH 7.4, cultures grown at 30° for 22 days.

Organism	$\mu\text{g. N/ml. medium}$									
	Medium before growth				Medium after growth				*	
	NH_4^+	NO_2^-	Uninoculated controls	NO_3^-	NH_4^+	NO_2^-	NO_3^-	Increase in NO_2^- -N in NO_2^-	Decrease in NH_4^+ -N in NH_4^+	% NH_4^+ lost, and recovered as NO_2^-
8 ₅	28	<0.048	<0.008	<0.008	17	8.9	<0.008	8.9	11	80.9
10	28	<0.048	<0.008	<0.008	19	7.8	<0.008	7.8	9	86.6
14 ₉	28	<0.048	<0.008	<0.008	16	7.5	<0.008	7.5	12	62.5
54	28	<0.048	<0.008	<0.008	17	9.6	<0.008	9.6	11	87.2
N = nitrogen										

* The last column refers to percentage recovery of the decreased ammonia nitrogen, as obtained before and after growth of the organism in the culture medium, which possibly then appears as nitrite nitrogen.

it is evident that these bacteria do produce nitrite in the presence of ammonia and in the absence of nitrate. Optimum conditions for growth and nitrite production in synthetic media have not been established.

Table 2. *Nitrite produced by two bacterial cultures, 8₅ and 14₉, grown in soil extract medium*

Medium: 50 % soil extract containing 0, 0.01 or 0.03 % NH₄Cl and 6.6×10^{-3} M-phosphate buffer, pH 7.4, cultures grown at 30° for 10 days.

Organism	Concentration of NH ₄ Cl (%)		
	0	0.01	0.03
	$\mu\text{g. NO}_2^- \text{--N/ml.}$		
8 ₅	5.8	11	10
14 ₉	3	16	16
Medium control	0	0	0

Tests for nitrate were negative on all samples.

Table 3. *Nitrite production by cultures 8₅ and 14₉ when grown in a defined medium*

Defined medium: 0.5 % sodium acetate or 0.5 % glucose, 0.01 % NH₄Cl, 0.61 % Tris (hydroxymethyl) aminomethane buffer, pH 7.4, 0.01 % NaHCO₃, 0.06 % MgSO₄, a trace of CaCl₂ and phosphates. Cultures grown at 30° for 7 days.

Experiment	Organism	$\mu\text{g. NO}_2^- \text{--N/ml.}$		$\mu\text{g. NO}_3^- \text{--N/ml.}$ 3 and 7 days
		3 days	7 days	
1. Sodium acetate the carbon source	8 ₅	0.14	0.19	0
	14 ₉	0.10	0.19	0
	Medium control	0	0	0
	no growth			
2. Glucose the carbon source	8 ₅	0.12	0.19	0
	14 ₉	0.10	0.18	0
	Medium control	0	0	0
	no growth			

Resting-cell studies and nitrite production. Culture 14₉ was grown on nutrient agar slopes for 20 hr. at 30° and washed three times in sterile distilled water. Organisms and supernatant were negative for nitrite and nitrate by the methods previously described. A solution composed of 0.08 M-phosphate buffer and 0.01 % (NH₄)₂CO₃ was used. To a portion of the latter, 2 ml. of washed organisms (7×10^8 /ml.) were introduced. These were maintained in screw-cap test-tubes measuring 20 × 120 mm. and filled over three-quarters full with solution. Tubes were tightly sealed and samples placed in the 37° incubator. Small portions of the suspension and the control were removed at the same time interval to test for nitrite production. The control remained negative for nitrite and nitrate throughout the experiment. A positive nitrite test was obtained after 54 hr. of incubation of the resting-cell mixture. This was con-

sidered a 1 plus positive test as described in the methods section. By 194 hr. the qualitative test for nitrite was 2 plus positive. The test for nitrate was negative at both time intervals.

Other cultures known to produce nitrite in soil extract media were also tested. After washing, however, the organisms were suspended in $(\text{NH}_4)_2\text{HPO}_4$ instead of the $(\text{NH}_4)_2\text{CO}_3$ substrate. These suspensions were incubated as before. Tests for nitrite and nitrate were negative at all times on the control substrate. A positive reaction for nitrate was never obtained on the test samples. By the end of 71 hr. incubation suspensions from nine different bacterial preparations gave positive tests for nitrite ranging from slight to 2 plus reactions. Most of the positive nitrite tests were obtained after 39 hr. incubation but some of the reactions were only slight at that time. The amount of nitrite produced by resting organisms was never as great as the amount produced when the organisms grew in soil extract medium. Nitrite production seems to be greatly increased by growth of the bacterial agent, however resting-cell observations further the idea of nitrite production by heterotrophs since the possibility of contamination in the system with traces of nitrite or nitrate is reduced.

DISCUSSION

The data reported in Table 1 strongly suggest the conversion of ammonia to nitrite, since ammonia disappeared as nitrite was formed in the absence of nitrate. In one instance as much as 87 % of the removed ammonia nitrogen could be accounted for as nitrite nitrogen. These findings do not necessarily imply that the mechanism for conversion of ammonia to nitrite by heterotrophic bacteria is the same as that employed by autotrophic bacteria. We know that smaller amounts of nitrite are produced by heterotrophs than by autotrophs. A comparative investigation of the mechanisms of conversion of ammonia to nitrite by heterotrophic and autotrophic bacteria using labelled nitrogen techniques might be rewarding.

Micro-Kjeldahl analysis of soil extract indicates that ammonia is not the only nitrogenous constituent in soil extract. Unknown nitrogenous components as well as ammonia may play roles in nitrite formation. It is conceivable that ammonia could be incorporated into cell protein while another nitrogenous component, other than nitrate, might be converted to nitrite. At any rate, ammonia disappears as nitrite appears and nitrate is not present in the system either before or after conversion under the conditions studied.

Resting-cell observations, as well as growth studies in defined media, point towards the conversion of ammonia to nitrite. Under these conditions any possibility of nitrate contamination is eliminated.

It has not been shown that all the nitrite produced comes from ammonia in the soil extract medium. It may be that nitrite production by heterotrophic bacteria is a more inclusive process than that conducted by autotrophic bacteria. The fact that heterotrophic bacteria participate in nitrite formation in the absence of nitrate suggests that the process is more universal than was formerly suspected.

No mention has been made concerning the importance of this reaction as an energy source. The amount of nitrite produced by heterotrophic bacteria compared to that produced by autotrophic organisms would suggest that heterotrophic nitrification is not an important energy-yielding mechanism for these bacteria. However, since small Gram-negative rods of groups 2 and 3 are considered the most prevalent morphological types in soil (Taylor & Lochhead, 1938; Conn, 1917*a, b*, 1948) it may be that these heterotrophic bacteria contribute substantially to the nitrogen cycle in soil.

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The Amino Acid Metabolism of *Aspergillus flavus*

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SUMMARY: A complete quantitative amino acid analysis of the free amino acid fraction and the mycelial residue fraction of the mould *Aspergillus flavus* during different stages of growth has been made by the circular paper chromatographic technique. A qualitative analysis of the amino acid composition of the culture fluid has also been accomplished. The changes of the individual amino acids present in the various fractions on different days of incubation were studied and their implications discussed.

The introduction of elegant and specific methods of amino acid analysis (e.g. microbiological assay, paper chromatography) has accelerated the pace of research on the amino acid metabolism in moulds. Woolley & Peterson (1936, 1937*a, b*) isolated thirteen amino acids from *Aspergillus sydowi* and pointed out that only physical isolation was certain proof of the presence of any amino acid. Synthesis of aromatic amino acids, viz. tyrosine and tryptophan from inorganic N by *A. niger*, *Zygorhyncus moelleri*, *Aspergillus oryzae*, *A. terreus* and *Penicillium flavo-glaucum* was indicated by colorimetric tests (Skinner, 1934). Chromatography has been used to study the amino acid metabolism of *P. chrysogenum* (Narasimha Rao & Venkataraman, 1952; Pyle, 1954).

In the present paper are presented the results of an investigation of the amino acid changes taking place in *Aspergillus flavus* during different stages of growth. The technique of circular paper chromatography (Giri & Rao, 1952*a, b*) with different solvent systems (Rao & Wadhwani, 1954, 1955) was used for the qualitative characterization and a quantitative estimation of most of the amino acids.

METHODS

Cultivation of the organism. A number of 1 l. Pyrex conical flasks were plugged with non-absorbent cotton-wool and sterilized empty at 20 lb./sq.in. pressure for 30 min. After cooling, 150 ml. of Czapek-Dox medium was distributed to each flask and sterilized at 10 lb./sq.in. pressure for 15 min. A heavy spore suspension of *Aspergillus flavus* was inoculated into each of the flasks and incubated at room temperature (26-28°). The mycelia were separated from the culture liquids by filtration on a Buchner funnel. The culture fluids were stored in 250 ml. Erlenmeyer flasks in a refrigerator until analysis. The mycelia were washed free from culture fluid with minimal amounts of normal saline. The mycelial felts were squeezed between filter-papers to remove moisture and dried in a desiccator (over P₂O₅) to constant weight.

Preparation of samples

Free amino acid fraction. Dry mycelium (0.5 g.) was ground with 10 ml. of 70 % (v/v) ethanol in water in a glass mortar for about 30 min. The suspension was centrifuged and the clear supernatant collected in a conical flask. The solid residue was re-extracted thrice in the same manner. All the clear supernatants after centrifugation were pooled and evaporated under low pressure (10 mm. of mercury, 50°) to dryness and made up to 1 ml. with 10 % (v/v) isopropanol in water. The lots of mycelium harvested on different days were treated in the same manner, and the respective extracts were used for analysis of free amino acids.

Acid hydrolysates of mycelial residues. The residues left after extraction by 70 % (v/v) ethanol-water, were dried to constant weight in a desiccator and 0.2 g. samples hydrolysed by refluxing with 20 ml. 6N-HCl for 24 hr. The hydrolysate was evaporated repeatedly to dryness on a water-bath, with the addition of distilled water to remove the acid present in the solution. After evaporation to dryness the residue left was dissolved in 2 ml. of 10 % (v/v) isopropanol in water, allowed to stand for 15 min. and then filtered. This filtrate was used for the amino acid analysis of the mycelial residue.

Culture fluids after growth. This was collected after the different periods of growth by filtration on a Buchner funnel. The fluids were stored under a layer of toluene in the refrigerator. For analysis 100 ml. samples of these fluids were evaporated to dryness under low pressure, extracted with 95 % (v/v) acid ethanol (ethanol + water + HCl: 95 + 4.5 + 0.5) to obtain salt-free amino acids (Baliga, Krishnamurthy, Rajagopalan & Giri, 1955), evaporated to dryness and the residue made up to 2 ml. with 10 % (v/v) isopropanol in water. These solutions were used for the amino acid analysis of the culture fluids.

Nitrogen estimations of the three different fractions were made by the micro-Kjeldahl method.

Paper chromatography. The circular paper chromatographic technique developed by Giri & Rao (1952*a, b*) was used for the identification of amino acids.

Two-dimensional chromatography by the ascending technique of Williams & Kirby (1948) with butanol + acetic acid + water (40:10:50) and pyridine + water (80:20) as solvents for the first and second runs, respectively, was found useful for confirming the identity of some of the amino acids, e.g. γ -amino-butyric acid, tryptophan and other ninhydrin-positive substances not easily identified on the circular paper chromatogram.

Some of the amino acids were identified by specific tests carried out by the multiple sector technique on a circular chromatogram with the experimental solution spotted at the centre. Different sectors were cut from a completely irrigated and dried chromatogram and tested by either dipping in, or drawing through the appropriate reagent contained in a watch-glass. The special reagents were prepared according to Block, LeStrange & Zweig (1952), Smith (1953) and Jepson & Smith (1953).

The quantitative procedure for the estimation of amino acids was essentially the same as that described by Giri, Radhakrishnan & Vaidyanathan (1952,

1953). The modifications introduced by Rao & Wadhvani (1955) regarding the solvent systems and temperature control for separation of amino acids were adopted with advantage.

RESULTS

Free amino acid composition of the mycelium. The amino acid composition of the ethanol-soluble fraction of the mycelium of *Aspergillus flavus* on different days of incubation is presented in Table 1. Most of the wide array of amino acids were at maximum concentration on the fifth day of incubation, with a subsequent decrease. The very high concentrations of alanine, glutamic and aspartic acids are interesting since aspartic and glutamic acids are known to be important parent substances for a number of other amino acids.

Table 1. *Free amino acid composition of the mycelium of Aspergillus flavus on different days of incubation*

Amino acid	No. of days incubation					
	5	10	15	20	25	30
	g. amino acid/100 g. of dry mycelium					
Cystine	0.344	0.312	0.225	0.216	0.099	0.099
Lysine	0.304	0.096	0.090	0.075	0.060	0.036
Arginine	0.344	0.099	0.078	0.063	0.063	0.051
Aspartic acid	0.400	0.084	0.066	0.066	0.066	0.060
Serine	0.144	0.081	0.063	0.030	0.021	0.048
Glycine	0.144	0.051	0.033	0.024	0.018	0.018
Glutamic acid	0.544	0.138	0.096	0.042	0.027	0.048
Threonine	0.184	0.096	0.066	0.066	0.048	0.039
Alanine	0.880	0.240	0.198	0.171	0.141	0.129
Proline	0.200	0.093	0.096	0.078	0.075	0.075
Tyrosine	0.240	0.117	0.075	0.063	0.033	0.033
γ -Aminobutyric acid	0.224	0.051	0.048	0.042	0.018	0.033
Tryptophan	0.092	0.036	0.012	—	—	—
Methionine	T	T	T	T	T	T
Valine	0.344	0.117	0.066	0.048	0.042	0.027
Phenylalanine	0.120	0.066	0.045	0.033	0.033	0.033
Isoleucine	0.176	0.063	0.033	0.021	0.021	0.024
Leucine	0.240	0.084	0.045	0.033	0.033	0.012

T=present in traces only; — =absent.

Of the basic amino acids only lysine and arginine were present in high concentration; histidine was hardly detectable even by the highly sensitive Pauly's test. Lysine and arginine from a maximum concentration on the fifth day had dropped to a low value by the tenth day. The disappearance of these amino acids continued till the thirtieth day though at a slower rate.

Leucine, isoleucine, valine and glycine were at maximum concentration on the fifth day with a subsequent marked decrease by the tenth day. Valine showed the maximum change in concentration. The presence of proline in the free condition is worthy of note.

Of the aromatic amino acids, tryptophan and phenylalanine were in very low concentration, the amount of tyrosine being somewhat higher. Tryptophan had practically disappeared by the fifteenth day.

Of the sulphur-containing amino acids cystine and methionine, methionine occurred only in traces in the free condition in *Aspergillus flavus*. Certain ninhydrin-positive spots occupying the positions of methionine sulfoxide, methionine sulphone and cysteic acid were, however, detected on two-dimensional chromatograms.

Table 2. *Free amino acid composition of the mycelium of Aspergillus flavus on different days of incubation*

Amino acid nitrogen as percentage of the total soluble nitrogen.

Amino acid	No. of days of incubation					
	5	10	15	20	25	30
Cystine	4.99	13.43	12.68	15.16	9.10	9.87
Lysine	7.26	6.80	8.84	8.68	9.07	5.90
Arginine	13.76	11.76	12.14	12.22	15.96	14.03
Aspartic acid	5.25	3.34	3.36	4.19	5.48	5.41
Serine	2.37	3.95	4.02	2.39	2.20	5.41
Glycine	3.34	3.51	2.97	2.69	2.65	2.87
Glutamic acid	6.44	4.85	4.41	2.40	2.00	3.90
Threonine	2.69	4.17	3.74	4.66	4.44	3.92
Proline	3.81	4.17	5.65	5.73	7.18	7.79
Tyrosine	2.31	3.34	2.80	2.93	2.00	2.18
γ -Aminobutyric acid	3.79	2.56	3.16	3.43	1.91	3.82
Tryptophan	1.57	1.83	0.80	—	—	—
Methionine	T	T	T	T	T	T
Valine	5.12	4.10	3.81	3.45	3.95	2.75
Phenylalanine	1.27	2.07	1.84	1.68	2.25	2.38
Isoleucine	2.34	2.48	1.71	1.36	1.77	2.21
Leucine	3.19	3.31	2.32	2.13	2.79	1.10

T = present in traces only; — = absent.

To assess the relative concentration of each amino acid in the mycelial extract, in Table 2 the nitrogen in each compound is expressed as a percentage of total soluble nitrogen. Irrespective of the duration of incubation, cystine, lysine and arginine were in a comparatively high concentration. The values for cystine, threonine and proline increased up to the twentieth day.

Amino acid composition of the mycelial residue. The amino acid composition of the ethanol-insoluble residue of the mycelium of *Aspergillus flavus* is given in Table 3, where the nitrogen of each amino acid is expressed as percentage of total nitrogen of the mycelial residue. The results show that the amino acid make-up of the mycelial protein varied during the period of incubation. From Table 3 it can be seen that the amount of bound glutamic acid in the protein was at a maximum in the fifteenth-day samples; alanine was at a maximum in the fifth-day sample. The concentration of these amino acids showed a decrease up to the twenty-fifth day after which there was a slight increase. Valine, tyrosine, leucine and isoleucine were minimum by about the twentieth day. There was a remarkably high concentration of lysine on the fifth day. After a subsequent steep fall in concentration till the tenth day there was again an increase till the twentieth day, and then a decline till the thirtieth day. It

Table 3. *Amino acid composition of ethanol-insoluble residue of the mycelium of Aspergillus flavus on different days of incubation*

Amino acid nitrogen as percentage of total nitrogen of the mycelial residue.

Amino acid	No. of days of incubation					
	5	10	15	20	25	30
Cystine	4.89	9.60	10.78	9.26	9.80	9.73
Lysine	28.11	10.63	12.50	22.93	17.64	12.96
Histidine	+	T	T	T	T	T
Arginine	9.75	6.11	6.34	5.11	6.21	6.89
Aspartic acid	6.69	9.16	8.77	7.07	11.70	13.64
Serine	3.62	5.86	7.41	6.13	6.00	5.21
Glycine	4.74	6.79	6.50	7.06	6.57	6.31
Glutamic acid	4.28	5.86	7.36	5.70	6.27	6.12
Threonine	3.10	5.03	4.81	3.88	3.61	4.11
Alanine	7.84	6.47	6.43	5.95	5.00	5.85
Proline	3.81	5.97	4.97	4.76	4.23	3.98
Tyrosine	3.40	4.90	2.70	1.89	2.99	3.66
Methionine	T	T	T	T	T	+
Valine	3.51	6.06	5.98	4.82	5.03	7.02
Phenylalanine	1.49	1.88	1.80	1.14	1.06	1.05
Isoleucine	3.45	3.04	2.75	2.22	2.31	2.29
Leucine	4.38	3.72	3.73	3.39	3.52	3.49

T=present in traces only; +=present.

was further observed that the amount of arginine decreased from a maximum concentration on the fifth day till the twentieth day and then showed signs of increase till the thirtieth day. The concentration of aspartic acid, however, was found to be minimum by the fifth day. After attaining a secondary minimum on the twentieth day the concentration rose sharply reaching a maximum by the thirtieth day.

Amino acid composition of culture fluids. The amino acid composition of culture fluids is presented in Table 4. As the amino acid concentration was

Table 4. *Amino acids in the culture medium of Aspergillus flavus on different days of incubation*

Amino acid	No. of days of incubation					
	5	10	15	20	25	30
Lysine	—	—	—	—	—	+
Aspartic acid	+	+	+	+	+	++
Glycine	++	++	++	++	++	+
Serine	+	+	—	T	+	+
Glutamic acid	T	T	T	T	+	++
Alanine	+	—	—	—	—	+
Tyrosine	—	—	T	T	T	+
Leucines	—	+	T	—	—	+
A	—	—	—	+	+	++
B	—	++	—	—	—	—
C	—	+	—	—	—	—

++=very prominent; +=present; T=present in traces only; —=absent; A, B and C are three ninhydrin-positive unidentified substances.

too low to be measured, and as it assumed a yellowish brown colour as the organism was being incubated, this study was restricted to a qualitative examination. Of the eight amino acids detected (Table 4) aspartic acid and glycine occurred throughout the period and were at a maximum on the thirtieth day. Aspartic acid and glycine were in a much higher concentration as compared to the other amino acids in the culture fluids at all times. There was as well a direct correlation between the disappearance of tyrosine, tryptophan, phenylalanine and the development of colour in the medium. Three unidentified ninhydrin-positive compounds in the culture fluids were also detected on the two-dimensional chromatograms.

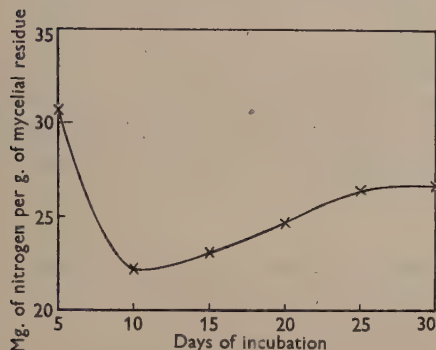


Fig. 1

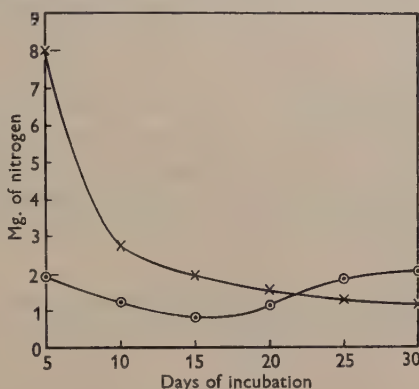


Fig. 2

Fig. 1. Changes in the nitrogen content of the ethanol-insoluble residue of the mycelium of *Aspergillus flavus*, on different periods of incubation. \times — \times , mg. of nitrogen/g. of mycelial residue.

Fig. 2. Changes in the nitrogen content of the ethanol-soluble fraction of the mycelium of *Aspergillus flavus* (\times — \times), and the changes in the total nitrogen content of the culture fluid of *A. flavus* (\circ — \circ) on different periods of incubation. \times — \times , mg. of soluble nitrogen/g. of dry mycelium; \circ — \circ , mg. of total nitrogen/100 ml. of the culture fluid.

The changes in the total nitrogen content of the ethanol-insoluble residue, ethanol-soluble fraction and the culture fluid of *Aspergillus flavus* on different periods of incubation are given in Figs. 1 and 2. The nitrogen content of the culture fluid was found to be much lower than in the other two fractions. The changes in concentration of nitrogen in the two fractions of the mycelium were more marked than in the culture fluid.

The total dry weight of the mycelium on different periods of incubation did not show any appreciable variation. However, it showed a slight increase from the fifth day to the fifteenth day, whereafter there was a decrease up to the thirtieth day of incubation.

DISCUSSION

The occurrence of high concentrations of amino acids in the ethanolic extracts of the fifth-day sample of the mycelium indicates that in the young mycelium there is intense metabolic activity, the amino acids synthesized being either

directly incorporated into the protein of the cells or used as a source of nitrogen for the synthesis of all the nitrogenous cellular constituents. From a consideration of the nitrogen content of both the ethanol-soluble and ethanol-insoluble fractions (Figs. 1, 2), it can be stated that the mycelium contains nearly 50 % more protein before prolific sporulation (fifth day) than after prolific sporulation.

The hydroxy amino acids serine and threonine were present in high concentration in bound form in the mycelial protein (Table 3), whereas their content in the ethanolic extract was low. This might be due to the efficient incorporation of these amino acids as soon as formed into the cellular protein. Information regarding the decarboxylation of amino acids by fungi is scanty. The detection of γ -aminobutyric acid, therefore, in the ethanolic extracts is interesting.

In contrast to the amino acid content of the mycelium, the culture fluid contained the maximum concentration of amino acids on the thirtieth day (Figs. 1, 2). This general increase in the concentration of amino acids and also of total nitrogen in the culture fluid (Fig. 2) towards the thirtieth day may be attributed to the proteolytic activity of the mycelium.

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Conversion of Cholesterol into Coprosterol by Bacteria *in vitro*

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SUMMARY: Certain anaerobic bacteria from human faeces were found to be able to hydrogenate cholesterol *in vitro*; some also decreased to a marked degree the total amount of sterols in the incubation mixture. Other organisms did not hydrogenate or decrease the amount of sterols under the conditions chosen; among these were; *Clostridium welchii*, *C. sporogenes*, *Bacterium bifidum*, various streptococci and micrococci, *Escherichia coli*, *Aerobacter aerogenes*. A 'germ-free' filtrate from human faeces was inactive. In a series of incubated samples, where the Tschugaeff reaction was applied to the non-saponifiable matter, an orange colour developed, suggestive of the presence of lathosterol.

That hydrogenation of cholesterol can be carried out with human faeces or colon contents *in vitro* was demonstrated by Dam (1934*b*). At the same time it was shown that faeces heated at 100° for 20 min. failed to hydrogenate cholesterol. Certain bacteria in pure culture, such as *Escherichia coli* and a type B enterococcus were inactive. Later, Rosenfeld, Fukushima, Hellman & Gallagher (1954*a, b*) succeeded in converting cholesterol into coprosterol with human faeces *in vitro*. Rosenheim & Webster (1943) found that the bacteriostatic agent succinylsulphathiazole inhibited coprosterol formation *in vivo* in rats, but they also observed inhibition by the amoebicidal agent *p*-carbamino-phenylarsonic acid which did not suppress the coliform flora. They therefore questioned the participation of bacteria in the process. The present work shows that cholesterol can be converted into coprosterol by some anaerobic bacteria from human faeces, whereas other such bacteria are inactive in this respect.

METHODS

Composition of media. Suspensions of brain were used as cholesterol-containing substrate. Rosenheim & Webster (1941) were of the opinion that cerebrosides such as those in brain are necessary for the hydrogenation of cholesterol in the intestine. However one of us (Dam, 1934*a*) found that, in man, faecal cholesterol was hydrogenated to more than 80% without ingestion of cerebrosides. Further, Rosenfeld *et al.* (1954*a, b*) used substrates without added cerebrosides in their *in vitro* experiments. The reason why we used brain suspensions as substrate is that such substrates are an easy means of providing a colloidal suspension of cholesterol which at the same time contains nutrients for bacteria. Desiccated hog or ox brain (2.5 or 5 g. portions) were stirred with tap water to make 5% (w/v) suspensions. In some cases, 1% (w/v) peptone and 0.1% (w/v) glucose were also added. Undissolved

matter was not filtered off. Sterilization was carried out for 30 min. at 100° (in live steam) on two consecutive days and for 30 min. at 120° on the third day. After the second heating 0.1 % (w/v) of cysteine hydrochloride was added. In every experiment the contents of all flasks were adjusted to contain the same amount of the substrate.

Flasks inoculated with clostridia were pasteurized at 80° for 7 min. after addition of the clostridia, but before the addition of non-sporing bacteria.

After inoculation as described in Table 1 the mixtures were incubated *in vacuo* in the dark at 37° for 7–8 days. After incubation the contents of each flask were saponified with an equal volume of 60 % (w/w) KOH on a steam bath for 2–3 hr. on 2 or more days until all bulky material had been dissolved. The non-saponifiable matter was shaken out with ether three times, the extract dried with anhydrous sodium sulphate, filtered, evaporated to dryness and the residue taken up in 25 ml. chloroform. The amount of dry matter in the chloroform solution was determined.

As a preliminary the Liebermann–Burchard reaction was carried out on all samples of the chloroform-soluble material. On some samples (Table 1, Exp. 4) Tschugaeff's reaction (see Hanel & Dam, 1955), was also used. Thereafter samples were taken up in 96 % (v/v) ethanol in water and the total amount of sterols determined by precipitation with digitonin (1 %, w/v, in 96 % ethanol). The determination with digitonin was repeated after adjustment of the concentration of the sterol as found by the preliminary determination, to 0.5 % (w/v), with a 50 % excess of digitonin.

The amount of saturated sterol was determined in another sample by the bromine-digitonin procedure of Schönheimer (1930) with the slight modification that, when possible, the final determination was made with an ethanolic (96 %) solution containing 0.5 % (w/v) of saturated sterol.

In cases where a degree of hydrogenation above 90 % was found, the saturated sterol was liberated from the digitonides by the pyridine-ether method (Schönheimer & Dam, 1933), recrystallized from 96 % ethanol and identified by crystal form, melting-point and melting-point of its mixture with a sample of coprosterol previously prepared from dog faeces.

RESULTS

The results are presented in Table 1. Expt. 1 shows (as found earlier: Dam, 1934*b*), that it is possible to hydrogenate cholesterol by incubation with human faeces *in vitro*. The same appears from Expt. 2*b* and *c*. In cases where the incubation had resulted in hydrogenation of the sterols, the non-saponifiable matter was oily or largely oily, whereas it was crystalline in cases where hydrogenation of sterols had not occurred. Expt. 2*f* shows that incubation with a 'germ-free' filtrate (i.e. free from dead organisms also) from the same faeces did not result in hydrogenation of cholesterol. Neither was hydrogenation obtained with a mixture of certain bacteria from the same faeces with or without addition of 'germ free' filtrate (Expt. 2*d*, *e*). The inactive bacteria were various streptococci, micrococci, *Escherichia coli*, *Aerobacter aerogenes* and *Bacterium bifidum*.

Table 1

TABLE 1

Inoculum	No. of days incubated at 37°	Total sterol (mg.)	Saturation of sterols (%)	Non-saponifiable material	
				mg.	Appearance
Expt. 1. 10% dried hog brain in water, total vol. 100 ml.					
(a) Human faeces 12.5 g. sterilized 120°, 15 min.	7	405	13.5	575	Crystalline
(b) Human faeces, 12.5 g.	7	350	80	690	Oily
Expt. 2. 5% dried hog brain +0.1% cysteine hydrochloride in water, total vol. 50 ml.					
(a) No addition	8	201	1.8	268	Crystalline
(b) 6 g. human faeces sterilized 120°, 15 min.	8	214	18	320	Crystalline
(c) 6 g. human faeces	8	198	82	340	Oily
(d) streptococci, micrococci, coli, aerogenes, bifidum from faeces of same person as above	8	199	18	305	Crystalline
(e) Bacteria as in (d) + 'germ-free' filtrate* from 6 g. human faeces	8	196	15.5	298	Crystalline
(f) 'Germ-free' filtrate* from 6 g. human faeces	8	196	4.0	290	Crystalline
Expt. 3. 5% dried ox brain +0.1% cysteine hydrochloride in water, total vol. 50 ml. (pH 7.2)					
(a) No addition	8	220	2.0	270	Crystalline
(b) <i>Clostridium welchii</i>	8	249	1.5	310	Crystalline
(c) <i>C. sporogenes</i>	8	258	2.0	306	Crystalline
(d) Anaerobic bacteria from human faeces†	8	136	89	385	Oily
(e) Anaerobic bacterium no. 103 from human faeces	8	229	2.2	252	Crystalline
(f) <i>B. bifidum</i> A9,‡ <i>B. bifidum</i> K1§	8	225	2.7	270	Crystalline
(g) Anaerobic bacteria nos. 101, 102, 106, 107 from human faeces	8	187	1.8	232	Crystalline
(h) Anaerobic bacterium no. 108 from human faeces	8	226	3.0	250	Crystalline
5% dried ox brain +0.1% cysteine hydrochloride in water, total vol. 50 ml. (pH 7.2) +1% peptone + 0.1% glucose					
(i) Anaerobic bacteria from human faeces	8	26	80	315	Oily
Expt. 4. 5% dried ox brain +0.1% cysteine hydrochloride in water, total vol. 100 ml. (pH 7.2)					
(a) No addition	8	465	2.1	570	Crystalline
(b) Clostridia nos. 3 and 7 from hamster faeces	8	465	5.0	620	Crystalline
(c) Clostridia M2 + H2 from human faeces	8	478	2.4	680	Crystalline
(d) Anaerobic bacteria from human faeces as in Expt. 3d	8	105	77.0	738	Oily
(e) Anaerobic bacteria from human faeces as in Expt. 3i	8	114	79.5	730	Oily
(f) Clostridia as in b and c + 16 different bacteria from human faeces	8	493	4.8	688	Crystalline
(g) Clostridia as in c + bacteria nos. 120, 121, 122 and 123 from human faeces	8	488	2.3	660	Crystalline
(h) Anaerobic bacteria tolerating 1 mg. Cu in 10 ml. substrate¶	8	267	93	773	Oily
(i) Bacteria as in c + h	8	260	93	800	Oily
(j) Culture as in d stored at 15°, 6 months	8	442	92	760	Oily
5% dried ox brain +0.1% cysteine hydrochloride in water, total vol. 100 ml. (pH 7.2) + 1% peptone + 0.1% glucose					
(k) Anaerobic bacteria from human faeces as in Expt. 3i	8	61.5	87	698	Oily

* The 'germ-free' filtrate from faeces was prepared by grinding 45 g. faeces with 90 g. water, centrifuging the mixture and passing the supernatant fluid through a sterile Seitz filter by suction.

† 5 g. faeces were inoculated into 25 g. 10% ox-brain extract + 0.1% cysteine HCl, and incubated *in vacuo* at 37° for 2 days; thereafter 0.5 ml. of the mixture was inoculated into another 25 g. portion of the same substrate, incubated *in vacuo* as above; thereafter the same procedure was repeated 3 times.

‡ Isolated from faeces of an old and presumably normal person.

§ Isolated from infant less than six months of age.

¶ As note † but + 1% peptone + 0.1% glucose in substrate.

¶ This mixture of anaerobic bacteria was obtained by inoculating the bacteria used in Expt. 3d into a 10% ox-brain extract to which had been added CuSO₄ in an amount corresponding to 1 mg. Cu/10 ml. medium. They were re-inoculated once into a similar substrate before use.

Expt. 3 shows that *Clostridium welchii* and *C. sporogenes*, two strains of *Bacterium bifidum*, and some anaerobic bacteria from human faeces did not hydrogenate cholesterol under the conditions of the experiment, whereas certain anaerobic bacteria from human faeces gave a high degree of hydrogenation (Expt. 3*d*, *i*). These latter bacteria also decreased the total amount of sterol in the incubation mixture, especially when peptone and glucose had been added (Expt. 3*i*). In Expt. 4*d*, considerable hydrogenation of cholesterol was obtained by the same anaerobic bacteria as in Expt. 3*d*. Expt. 4*e*, *k* show that these same bacteria also hydrogenated cholesterol, whether or not peptone and glucose had been added. When these anaerobic bacteria were grown in a medium containing 1 mg. Cu/10 ml. their ability to hydrogenate cholesterol remained intact.

Expt. 4*d* was repeated after the culture had been stored at 15° for 6 months. The results (Expt. 4*j*) show that the ability to hydrogenate cholesterol had been retained, whereas the ability to convert cholesterol to non-digtonin precipitable substances was largely lost.

Several clostridia and bacteroides from human faeces did not hydrogenate (Expt. 4*b*, *c*, *f*, *g*).

The digitonides from the Expt. 4*h*, *i* and *j* were dissolved in pyridine and decomposed with ethyl ether according to Schönheimer & Dam (1933), digitonides from *h* and *i* being pooled. After recrystallization from 96% ethanol the sterol obtained from (*h* + *i*) melted at 98–100°, that from *j* at 99–101°. In both cases the crystals showed the needle form characteristic of coprosterol and showed no depression of melting-point after being mixed with a sample of coprosterol obtained from dog faeces. Attempts are being made to isolate, from the mixtures which were found active, purified cultures of organisms capable of hydrogenating cholesterol. In these mixtures *Bacteroides* spp. and pleuropneumonia-like organisms were especially evident.

In Expt. 4, where the Tschugaeff reaction was used, it was found that samples *c*, *d*, *e*, *h*, and *k* gave an orange colour, suggesting the presence of lathosterol (Δ^7 -cholestenol; cf. Hanel & Dam, 1955). This recalls the finding of lathosterol in rat faeces reported by Wells, Coleman & Bauman (1955).

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The Virulence of Biochemical Mutants of *Erwinia aroideae* for Varieties of Radish and Turnip

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SUMMARY: Biochemical mutants of *Erwinia aroideae* displayed a pattern of virulence and avirulence for varieties of radish and turnip similar to the pattern previously reported for different host species. Three types of host response were noted when slices of the fleshy storage organs were inoculated: uniformly resistant, uniformly susceptible, and a variable response in which individuals of a given variety may be resistant or susceptible to a specific mutant. Prototrophic reversions from an avirulent mutant requiring arginine and from an uncharacterized mutant with diminished virulence were as virulent as the parental strain for all varieties of radish and turnip.

Pathogenicity may be defined as a specific expression of a host-parasite relationship in which the host serves as an environment for the parasite. The property of pathogenicity with respect to the parasite assumes that the parasite is capable of utilizing the host environment as a growth medium and that the parasite can overcome the host's defence mechanisms. It should be noted that two problems are involved: the ability of the parasite to proliferate or to metabolize extensively and mechanism(s) whereby the parasite damages the host either *in toto* or in specific tissues or cells of the host. The first problem has been relatively neglected. The role of nutrition in the host-parasite relationship may be referred to the nutritional requirements of the parasite as they may or may not be satisfied by the host environment.

Recent studies with biochemical mutants of animal and plant pathogens have indicated that mutants with specific nutritional requirements may be avirulent if the host environment does not supply an adequate concentration of the required nutrilites, either at the site of inoculation or of localization (Bacon, Burrows & Yates, 1951; Garber, Hackett & Franklin, 1952; Garber, 1954; Keitt & Boone, 1954). Garber (1954) observed that certain biochemical mutants of *Erwinia aroideae* were not uniformly virulent for a given host species, and assumed that the variation in virulence may have resulted from the inadvertant usage of different varieties of the host species. This report presents data on the virulence of biochemical mutants of *E. aroideae* for a number of varieties of radish and turnip.

METHODS

Erwinia aroideae, strain RK (Garber & Hackett, 1954), is a bacterial plant pathogen producing a soft rot in the fleshy storage organs of numerous vegetable species. Organisms were grown in A medium (Davis & Mingioli, 1950) at 27° with constant shaking for 24 hr. The culture was serially diluted in

0.8 % (w/v) NaCl to yield a suspension containing $1-4 \times 10^4$ viable organisms/ml. Ten ml. of this suspension were placed in a sterile Petri dish and irradiated for the desired length of time by means of a General Electric 'Sterilamp' (15 W.) at a standardized distance so that the dosage was approximately 300 ergs. The dish was agitated gently during the period of exposure. Samples (0.1 ml.) of irradiated organisms were inoculated into 9 ml. of nutrient broth (Difco), supplemented with 0.1 % yeast extract (Difco) and incubated at 27° with constant shaking for 24 hr. with suitable precautions against photoreactivation (Kelner, 1949). The cultures were centrifuged, washed with 0.8 % NaCl twice, resuspended in A medium containing 300 units of penicillin/ml., and incubated at 27° for 24 hr. Samples (0.1 ml.) were then plated on nutrient agar (Difco) supplemented with 0.1 % yeast extract and incubated at 27° for 48 hr. Colonies selected at random were picked and inocula were added to a plate containing A medium solidified with Noble agar (Difco) and to a plate containing nutrient agar supplemented with yeast extract. Colonies failing to grow on the A medium were retested on this medium. Nutritional requirements were determined auxanographically (Pontecorvo, 1949) using commercially available amino acids, vitamins, growth factors, purines, and pyrimidines. Once a mutant was characterized, no attempt was made to establish the specific site or nature of the nutritional block. Thirty biochemical mutants were isolated, all but one requiring one or more amino acids. Four mutants were not characterized completely but they required one or more amino acids.

To prepare inocula for the virulence tests, the parental and mutant strains were grown in nutrient broth supplemented with 0.1 % yeast extract at 27° with constant shaking for 24 hr. The cultures were centrifuged, washed with 0.8 % NaCl, and resuspended in 0.8 % NaCl to yield a suspension containing $1-4 \times 10^9$ viable organisms/ml. These suspensions were then incubated at 27° with constant shaking for 4 hr. The surface of slices of the fleshy storage organs of the host was inoculated with 0.05 ml. of this suspension.

Seed of nine varieties of radish and three varieties of turnip were purchased from the W. Atlee Burpee Co., Philadelphia, Pennsylvania, U.S.A., who also provided the varietal names. Mature plants were harvested and the fleshy storage organs were washed with tap water, immersed in a 20 % (v/v) solution of 'Chlorox' for 3-5 min., and rinsed with sterile, distilled water. The fleshy organs were then sliced with a sterile knife, the slices being placed in a sterile Petri dish containing a saturated layer of filter-paper. The slices were inoculated by dropping the bacterial suspension on the upper surface and then incubated at 27° for 24 hr. A slice from at least three different plants of each variety was used in routine tests of virulence and, in some experiments, as many as nine plants were used. After incubation, slices which had been attacked displayed a discoloured, slimy, glistening surface which was easily penetrated by a blunt, glass rod; slices which had not been attacked remained white and firm. Suitable controls were routinely used. Since preliminary experiments consistently yielded cells of the inoculated strain, re-isolation of the mutants from attacked slices was not routinely done.

Three types of response were noted when plants of a given variety were inoculated with the biochemical mutants: uniformly resistant, uniformly susceptible, and a variable response, i.e. some resistant and some susceptible individuals in a given variety. Slices from a single, fleshy organ displayed a uniform response when inoculated with a given biochemical mutant.

RESULTS

The radish as host. Relatively few mutants appeared to have lost their virulence for the radish (Table 1). Both mutants requiring arginine were avirulent for all nine varieties. The four mutants which were not fully characterized displayed either avirulence or a greatly diminished virulence for all nine varieties. The mutants requiring isoleucine and valine exhibited a striking pattern of virulence and avirulence. Although the mutants requiring cysteine, histidine or glycine also showed a pattern of virulence and avirulence, the differences among these mutants were relatively minor.

Since it had been demonstrated that prototrophic reversions from an avirulent biochemical mutant of *Erwinia aroideae* would be virulent (Garber & Hackett, 1954), a similar demonstration was attempted with a mutant requiring arginine (21-1-1) and a mutant (35-7-57) which had not been fully characterized. Two independent prototrophic reversions from mutant 21-1-1 and three independent prototrophic reversions from mutant 35-7-57 were as virulent as the parental strain for all nine varieties.

The possible effect of a high reversion rate on the virulence tests was determined. Mutants which were virulent for all nine varieties were tested for their frequency of prototrophic reversions by plating $c. 1 \times 10^8$ viable organisms on solidified A medium. Only mutant 19-8-40 requiring cysteine and mutant 20-1-51 requiring methionine had a high frequency of reversions. Repeated virulence tests using numbers of viable organisms of these mutants, adjusted to make unlikely the inclusion of reversions, confirmed the original observations that these mutants were uniformly virulent for all varieties.

Mutants displaying a variable virulence for certain varieties were further tested by inoculating slices from additional plants of these varieties. The results summarized in Table 2 indicated that not all plants in these varieties responded uniformly. In some combinations of mutant and variety, $c. 50\%$ of the plants were resistant and the remainder, susceptible.

The turnip as host. The pattern of virulence and avirulence of the mutants for the three varieties of turnip paralleled to a considerable extent the pattern noted for the radish varieties (Table 3). Both mutants requiring arginine were avirulent for all three varieties. The four mutants which could not be fully characterized likewise were avirulent with a single exception. Whereas the mutants requiring cysteine or glycine remained comparatively virulent, the mutants requiring histidine or isoleucine and valine were greatly diminished in their virulence. Specific mutants able to attack many radish varieties were not virulent for all the turnip varieties.

As was the case in the virulence tests using the radish varieties, certain

Table 1. *The virulence of biochemical mutants of Erwinia arorideae for nine varieties of radish*

Mutant	Need	Varieties								
		White icele	White	Comet	Scarlet globe	Crimson giant globe	Red giant	Rapid red	Sparkler	French breakfast
21-1-1	Arginine	0	0	0	0	0	0	0	0	0
1-3-50	Arginine	0	0	0	0	0	0	0	0	0
38-2-48	Cysteine	+	+	+	+	+	+	+	+	+
38-16-20	Cysteine	+	+	+	+	+	+	+	+	+
43-1-20	Cysteine	+	+	+	+	+	+	+	+	+
43-1-21	Cysteine	+	+	+	+	+	+	+	+	+
19-8-40	Cysteine	+	+	+	+	+	+	+	+	+
36-3-48	Cysteine	+	+	+	+	+	+	+	+	+
36-9-46	Histidine	+	+	+	+	+	+	+	+	+
19-24-26	Histidine	+	+	+	+	+	+	+	+	+
35-8-49	Histidine	+	+	+	+	+	+	+	+	+
36-1-12	IV	+	+	+	V	+	+	+	V	V
18-3-52	IV	+	+	+	V	+	+	+	V	0
35-9-12	IV	+	+	+	+	+	+	+	V	0
35-8-32	IV	+	+	+	+	+	+	+	V	V
36-1-38	IV	+	+	+	+	+	+	+	0	V
42-10-16	Tryptophan	0	+	+	0	0	V	0	0	0
36-10-59	Phenylalanine	+	+	V	+	+	+	0	0	0
43-2-33	Glycine	+	+	+	+	+	+	V	V	0
43-3-6	Glycine	+	+	+	+	+	+	+	+	+
43-2-30	Glycine	+	+	+	+	+	+	+	+	+
43-2-6	Glycine	+	+	+	+	+	V	+	+	+
20-1-51	Methionine	+	+	V	+	+	V	+	V	+
13-8-21	Threonine	+	+	+	+	+	+	+	+	+
18-2-21	Leucine	+	+	+	+	+	+	+	+	+
42-18-57	Asparagine	+	+	+	+	+	+	+	+	+
40-8-25	Unknown AA	+	0	0	+	+	+	+	+	0
35-7-57	Unknown AA	+	0	V	0	V	0	0	0	0
34-3-36	Unknown AA	0	0	0	0	0	0	V	0	0
43-2-37	Unknown AA	0	0	0	0	0	0	0	0	0

+ = uniformly virulent; 0 = uniformly avirulent; V = variable. IV = isoleucine and valine; unknown AA = one or more amino acids.

Table 2. *The response of individual plants of varieties of radish to certain biochemical mutants of Erwinia aroideae*

Mutant	Need	Varieties													
		Comet		Scarlet globe		Crimson Giant globe		Red giant		Rapid red		Sparkler		French breakfast	
		S	R	S	R	S	R	S	R	S	R	S	R	S	R
36-9-46	Histidine	6	2	5	4
35-8-49	Histidine	.	.	4	5	3	6	.	.
36-1-12	IV	.	.	3	5	5	3	5	4
18-3-52	IV	4	5	.	.
35-9-12	IV	5	4	.	.	3	5	.	.	2	6
35-8-32	IV	2	6
42-10-16	Tryptophan	4	5	7	2	5	3	.	.
43-3-6	Glycine	1	2
43-2-30	Glycine	4	5
43-2-6	Glycine	4	2	5	1	.	.
13-8-21	Threonine	6	2	.	.
35-7-57	Unknown AA	4	1	.	.	4	5
34-3-36	Unknown AA	1	4

S=susceptible; R=resistant. IV=isoleucine and valine; unknown AA=one or more amino acids.

Table 3. *The virulence of biochemical mutants of Erwinia aroideae for three varieties of turnip*

Mutant	Need	Varieties		
		Extra early purple top Milan	Purple top white globe	Foliage
21-1-1	Arginine	0	0	0
1-3-50	Arginine	0	0	0
38-2-48	Cysteine	+	+	+
38-16-20	Cysteine	+	+	+
43-1-20	Cysteine	+	+	+
43-1-21	Cysteine	+	+	+
19-8-40	Cysteine	+	+	+
36-3-48	Cysteine	V	0	0
36-9-46	Histidine	+	0	0
19-24-26	Histidine	+	0	0
35-8-49	Histidine	+	0	0
36-1-12	IV	V	V	V
18-3-52	IV	V	0	0
35-9-12	IV	+	V	0
35-8-32	IV	V	V	0
36-1-38	IV	V	0	0
42-10-16	Tryptophan	+	+	V
36-10-59	Phenylalanine	+	+	+
43-2-33	Glycine	+	+	+
43-3-6	Glycine	+	+	+
43-2-30	Glycine	+	+	+
43-2-6	Glycine	V	+	+
20-1-51	Methionine	+	+	+
13-8-21	Threonine	V	0	0
18-2-21	Leucine	+	+	V
42-18-57	Asparagine	+	+	+
40-8-25	Unknown AA	0	0	0
35-7-57	Unknown AA	V	0	0
34-3-36	Unknown AA	0	0	0
43-2-37	Unknown AA	0	0	0

+ = uniformly virulent; 0 = uniformly avirulent; V = variable. IV = isoleucine and valine; unknown AA = one or more amino acids.

combinations of mutant and turnip variety yielded a variable virulence. Further tests with these combinations confirmed the original observations (Table 4).

Table 4. *The response of individual plants of varieties of turnips to certain biochemical mutants of Erwinia aroideae*

Mutant	Need	Varieties					
		Extra early purple top Milan		Purple top white globe		Foliage	
		S	R	S	R	S	R
36-3-48	Cysteine	5	4
36-1-12	IV	5	3	5	3	.	.
18-3-52	IV	7	2	.	.	3	6
35-9-12	IV	.	.	5	3	.	.
35-8-32	IV	5	3	3	6	.	.
36-1-38	IV	3	5
42-10-16	Tryptophan	4	5
13-8-21	Threonine	4	5
18-2-21	Leucine	7	2
43-2-6	Glycine	5	1
35-7-57	Unknown AA	7	2

S=susceptible; R=resistant. IV=isoleucine and valine; unknown AA=one or more amino acids.

DISCUSSION

The biochemical mutants of *Erwinia aroideae* exhibited a pattern of virulence and avirulence for varieties of radish and turnip similar to that previously reported for different host species (Garber, 1954). This pattern which had been interpreted as an expression of the relationship between the demand for required nutrilites by the parasite and the supply of these nutrilites by the host, either at the site of inoculation or of localization, may now be extended from the species level to the variety level. It should be emphasized that an 'inadequate' supply does not imply an absence of the required nutrilites (Garber, 1954). The resistance or susceptibility of varieties or species to specific biochemical mutants may reflect different concentrations of the required nutrilites in the hosts. A similar interpretation may be offered for the variable response of individuals in a variety. Supporting evidence must come from an *in vitro* demonstration of different concentrations of the required nutrilites (in the amino acid content) among the varieties of radish and turnip.

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The Streptococci of Lancefield's Group E; Biochemical and Serological Identification of the Haemolytic Strains

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SUMMARY: The non-pathogenic beta-haemolytic streptococci from milk, forming Lancefield's group E, were physiologically defined as *Streptococcus infrequens* and *S. subacidus*. They were found to fall into four serological types. Fractionation with ethanol showed that both group- and type-specific fractions of these organisms are of polysaccharide nature which probably accounts for the success in using formamide extracts for the type-precipitin reactions. *S. subacidus* possesses two type antigens, only one of which is also found in *S. infrequens*.

Little attention has been paid to serological group E since Lancefield identified its group-specific antigen (1933). Isolation of several strains was reported by Newson (1937), Gunnison, Luxen, Marshall & Engle (1940), Plummer (1941), Stafseth & Clinton (1941), Coffey (1942), and, more recently, by Collier (1951), Thal & Moberg (1953) and others. However, no substantial contribution has yet been made either to the type division of this group or to the chemical nature of the antigenic fractions responsible for its serological pattern. *Topley & Wilson's Principles* (1946) mention, in a table, that both type and group substances are polysaccharides; also that several specific types have been recognized, but reference is not made to the work leading to such findings. The only relevant literature found was a personal communication of Lancefield to Stafseth & Clinton (1941) in which she admitted the existence of more than one type.

METHODS

Source of cultures. Altogether twenty haemolytic streptococci were collected for study. I am indebted to Mr F. K. Neave for six strains (with the suffix E) isolated during the course of this investigation; to Dr Julia Coffey for six strains (with the suffix CO) isolated during the period 1934 to 1942; to Dr H. J. Stafseth for the 'Newson' strain. The remaining seven strains were stock cultures kept at the National Institute for Research in Dairying; these included strains K 128, K 129 and K 131 isolated about 1925 by Dr J. A. Brown (Lancefield, 1933) and two strains, C 24A and C 24B isolated by Dr Stafseth and received from Dr Lancefield. The streptococci were obtained from milk with the following four exceptions: 4051CO, isolated in 1940 by Dr Julia Coffey (personal communication) from drainage of wound on the hand of a dairy worker; 'Newson' isolated from a cervical suppurating lymph gland of a pig (Newson, 1937); C 24A and C 24B isolated from abscessed lymph nodes of swine by Dr H. J. Stafseth.

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Biochemical and cultural tests

Haemolysis was tested by streaking 18 hr. cultures on 5 % horse-blood agar with a basal medium of 2 % (w/v) agar, 1 % peptone (Evans), 1 % Lab-Lemco and 0.5 % NaCl. The plates were incubated for 48 hr. at 37°.

Reducing properties. Reactions in litmus milk and methylene blue milk (1:5000) were recorded after 5 days of incubation at 37°, with preliminary readings after 24 and 48 hr.

Carbohydrate fermentations. Seitz-filtered 0.5 % (w/v) 'sugar' solutions (except aesculin) were added to peptone water containing 1 % Andrade's indicator and 1 % ox serum.

Hydrolysis of sodium hippurate. This test was carried out as applied by Davis, McClement & Rogers (1939). A further modification was made: 1 ml. of the sterile hippurate broth was added to tubes with progressive numbers of drops of 12 % FeCl₃ solution containing 2.5 ml. of concentrated HCl/l.; after agitation, the first clear tube in the scale was noted. The presence of benzoic acid was then determined by adding 1 ml. of culture to the recorded number of drops of reagent.

Growth at 45°. Tubes of glucose 1 % Lemco broth and yeast glucose litmus milk were seeded, incubated in a 45° (± 0.1) water-bath and examined for growth after 24 hr.

Survival at 60° for 30 min. Tubes of glucose Lemco broth were warmed at 55–59°, inoculated and held for exactly 30 min. in a water-bath at 60° (± 0.1), cooled immediately and incubated at 37° for 24 hr.

Growth at pH 9.6. The technique of Shattock & Hirsch (1947) was used.

Inoculations. All the liquid media were seeded with 1–2 drops of an 18 hr. culture. Readings were made on the fifth day of incubation.

Serological methods

The medium generally used for all serological purposes was glucose (0.5 %) Lemco broth.

Preparation of antisera. Cocci suspended in saline were heat-killed at 57° for 30 min. or killed in 0.25 % formalin (Lancefield, 1933). Cocci twice washed in acetone and dried in vacuum over P₂O₅ were then ground in a ball mill under cold water (Shattock & Mattick, 1943). The stock vaccines were diluted to the opacity of Brown's tube no. 7 (Burroughs Wellcome & Co.), fresh dilutions being made for each injection. Inoculations were performed every 3 or 4 days, the first dose being 0.5 ml., the second 1 ml., and thereafter 1.5 ml.

Precipitin test

Formamide extracts. Fuller's (1938) method was carried out by extracting the cocci of 5 ml. culture with 1.0 ml. formamide at 150° for 15 min.; 2.5 ml. acid ethanol was added, the resulting precipitate discarded and the clear supernatant mixed with 5.0 ml. acetone. The precipitate formed was dissolved in 1.0 ml. saline.

Crude HCl extracts. Sediments of 100 ml. cultures were extracted in 2.5 ml. 0.05N-HCl in saline (Lancefield, 1933). Extracts resistant to clarification by spinning were treated with a few drops of trypsin solution and incubated for 2 hr., boiled for 5 min. and then centrifuged. This was the most successful method among several tried.

Antigenic fractions. Pure solutions of group and type substances were obtained by extracting the twice-washed sediments with 0.05N-HCl in saline. The organisms were attacked a second time with 0.2N-HCl in distilled water, the stronger acid solution being applied to overcome the increased cellular resistance. To both first and second crude extracts, neutralized, centrifuged and kept separated, were added four volumes of ethanol and then they were stored in the cold. After centrifugation, the ethanolic supernatants were kept in the cold overnight and the protein precipitate dissolved in 2.0 ml. saline and reprecipitated with three volumes ethanol, ethanol washings being repeated until the Molisch reaction was negative. The combined supernatants were evaporated and the dried C-substance treated with ethanol until the biuret reaction was negative. When finally separated, both fractions from 1 l. cultures were dissolved in 3.0 ml. saline, neutralized, centrifuged and kept in the cold with 1/10 (v/v) of 5% carbol-saline.

Specific precipitation sera. Non-specific group or type antisera were absorbed respectively with group or type cross-reacting strains. One volume of packed heterologous cells, heat or formol killed, was emulsified with two/three volumes of undiluted serum and the suspension left in the water-bath at 37° for 30 min., repeatedly shaken and centrifuged. The sediment was left in the tube and, if proper absorption was not achieved, the cocci were again emulsified, the mixture immersed in the water-bath for 2 hr. and left overnight in the cold. When heterologous antibodies were not eliminated, another cross-reacting strain was tried with a fresh volume of antiserum.

Precipitin reactions. The extract was layered on the antiserum in a precipitin tube of 2 mm. internal diameter and the rings recorded within 1 to 5 min.

The type-agglutinin reaction

Water-bath agglutination. Dreyer's technique was followed using equal amounts (10 drops) of reagents, the tubes being left in a water-bath at 50° and read after 4 and 18 hr. Cultures in glucose (0.1%) Lemco broth, usually 16 hr. old, containing 20,000–40,000 $\times 10^6$ organisms/ml., and killed by formalin (0.25%) were used as antigens. Auto-agglutinability was generally overcome by heavy seeding and incubation for 6–8 hr. with occasional shaking, a method which proved the most suitable among several tried.

Slide agglutination. For this test, serum was diluted to 1/32 of its (water-bath) titre. A 'slide end-titre' was also used following De Waal's procedure (1940); serum was added drop by drop to 2.0 ml. carbol-saline until good clumping was observed within 10–20 sec. with the homologous suspension. Sera of low agglutination content were dropped into 1 ml. carbol-saline. Bacterial antigens were suspensions of cocci in carbol-saline, with an opacity of roughly five times Brown's tube no. 7; denser opacities tended to make

readings difficult. Reactions appearing later than 2 min. (only seldom observed) were taken as non-specific. Spontaneous granularity was sometimes successfully prevented by incubating the cocci with a few drops of trypsin solution until instability could no longer be observed.

Specific agglutinating antisera. The heterologous agglutinins were eliminated by mixing undiluted serum with packed organisms of the absorbing strain in a manner similar to that applied to sera for the precipitin test, the end-titre dilution being obtained by the technique of De Waal (1940). In a second method, the serum was first diluted to 1/64 of its titre and mixed with an equal quantity of a suspension of formol-killed organisms in carbol-saline with an opacity 10 times that of Brown's tube no. 7. The tube was incubated in a 37° water-bath for 30 min., 2 hr. or left overnight in the cold if cross-reactions were not eliminated by the first two treatments. The actual dilution of the serum was 1/32 of its titre.

RESULTS

Biochemical and physiological reactions

Seventeen of the twenty strains collected for this study formed a group (subsequently referred to as *Streptococcus infrequens*) with the following characters (Table 1): fermentation of sucrose, salicin, mannitol, sorbitol, trehalose and hydrolysis of aesculin; no action on inulin and raffinose; litmus milk was generally acidified, but five strains were able to coagulate the medium with slight reduction; the action on methylene blue milk was irregular, and nine strains reduced the dye, four of them with further coagulation; the reaction on sodium hippurate was negative in all but three strains which slightly hydrolysed this substance. The three remaining streptococci (referred to as *S. subacidus*) were differentiated from this group because of their failure to split aesculin, mannitol and salicin; the fermentation of sucrose was irregular; they had no action on methylene blue milk and only slightly acidified litmus milk. Beta-haemolysis with wide and clear zones was produced by the surface colonies of all strains after 48 hr. incubation. The colonies did not exhibit any distinctive characteristic except those of the second group which were of 'minute' size. All the strains produced good growth in liquid media after 24 hr. incubation at 37°, but 4678 was exceptional in requiring at least 48 hr. incubation, perhaps because of the small size of its cocci.

Group reactions

Extracts of all the strains have given precipitin (ring) reactions with sera of group E. Similar positive results were obtained either with the ordinary crude HCl or formamide extracts. Experience showed that it is also possible to extract these streptococci with 0.2N-HCl in distilled water, a technique applied by Swift, Wilson & Lancefield (1943) to group A streptococci in order to get stronger concentrations of M substance for type precipitation. These extracts were prepared in the ordinary way except that, for final neutralization, a solution of 1% Na₂HPO₄ in 0.2N-NaOH, balanced against 0.2N-HCl, was used. This buffer solution provides a suitable salt concentration for the precipitin

Table 1. *Physiological and serological reactions*

Strains	Haemolysis	Litmus milk reaction	Growth in methylene-blue milk (1:5000)	Action on carbohydrates								Growth at 45°	Growth at pH 9.6	Survival at 60°, 30 min.
C24A	β	A	-	Sucrose	+	+	+	+	+	+	+	+	+	+
C24B	β	A	pR	Salicin	+	+	+	+	+	+	+	+	+	+
TY7	β	ApCR	-	Mannitol	+	+	+	+	+	+	+	+	+	+
K129	β	A	-	Inulin	-	-	-	-	-	-	-	-	-	-
K131	β	A	-	Raffinose	-	-	-	-	-	-	-	-	-	-
28E	β	A	-	Sorbitol	+	+	+	+	+	+	+	+	+	+
30E	β	A	R	Trehalose	+	+	+	+	+	+	+	+	+	+
35E	β	A	CR	Aesculin	+	+	+	+	+	+	+	+	+	+
40FE	β	A	-											
40HE	β	A	pR											
R10E	β	ApCR	-											
4051CO	β	AC	pR											
4211CO	β	ACpR	CR											
34497CO	β	A	R											
41483CO	β	A	CR											
41484CO	β	AC	-											
Newson	β	A	-											
K128	β	Sl.A	-											
4678	β	Sl.A	-											
34498CO	β	Sl.A	-											

A = acid; Sl.A = slight acid; R = complete reduction; pR = partial reduction; C = clot; + = acid.

reaction, and in fact the required amount of electrolyte is so small that precipitin rings could even be produced in distilled-water extracts without the addition of buffer. Formamide extracts, either from 5 ml. (Fuller's original technique) or from 10 ml. culture were also successful. Ring precipitations could still be obtained in extract dilutions of 1/5 to 1/20 (v/v).

Cross-reactions reported by Lancefield (1933) between her strain K131 and a group C antiserum could not be confirmed; all the extracts were negative against an antiserum of that group. It was also found that the physiological relationship between the enterococcus and group E (sorbitol and trehalose are fermented by both groups) was not reflected in serological relationship and all the E extracts failed to react with a group D antiserum prepared from a haemolytic strain. The group D antiserum mentioned in this work was obtained from *Streptococcus durans* strain 98D with a suspension of disintegrated (ground) cocci.

Characteristics of group E antisera. Altogether, nine rabbits were inoculated with different suspensions for various periods of time. Serum 273, prepared with formolized cocci of C24B, showed very strong group-specific antibody content after the fourth injection. Serum 294 was prepared against 4678, also formol-killed. In the four bleedings, only type-specific antiserum was obtained, but group reactions appeared after thirty inoculations. Immunization was continued, but, in all subsequent bleedings, type-specificity appeared again. Strain TY7 was chosen for the preparation of the third serum, 227; a suspension made with disintegrated cocci was used, but no response could be obtained in the first three bleedings; after thirty-eight inoculations, the serum showed only strain specificity. It was decided to change the type of suspension and group antibodies appeared after nine inoculations with heat-killed cocci. However, during subsequent immunization, the antiserum again became type-specific and group specificity was not recovered during the course of forty further injections. In the late stages of this work, two other formolized vaccines were prepared from the freshly isolated 28E and 4211E; their respective antisera, 311 and 312, showed only type-specific antibodies. Lancefield strains K131 and K129 gave sera which cross-reacted with several extracts of groups A-N and also with the nucleoprotein P. Absorption with heavily packed staphylococci did not render these sera specific.

Type reactions

It was possible to divide the strains into three well-defined types, a fourth type being formed by the strain 'Newson' which did not react with any of the available antisera. Type differentiation, with parallel results, was carried out by the precipitin reaction accompanied by slide and water-bath agglutinations. Acid extraction, either by 0.05N- or 0.2N-HCl as well as formamide extraction proved to be satisfactory (Table 2). Some discrepancies arising from the different antibody content of the antisera will be recorded in detail.

Type I. Into type I fell Lancefield strains C24A, C24B, K129 and also the K128. Strain K128 showed auto-agglutinability resistant to several forms of treatment, but a stable suspension was obtained with cocci treated with

trypsin. Two antisera were available: serum 273, prepared against C 24 B, and serum 250, prepared against K 129. The titre of both sera was 1/2560. Before being satisfactorily absorbed by strain TY 7 the first showed complete group specificity, whereas the second gave positive precipitin reactions with nucleoprotein P; however, both were proved type-specific by the agglutinin tests. The four strains falling into type I exhausted both sera of their homologous antibodies. The HCl extract of type II strain 28 CO produced a slight cross-reaction with antisera of type I which was not observed with the corresponding formamide extract.

Table 2. *Type division of haemolytic strains of group E*

Strains	Types			
	I	II	III	IV†
C 24 A	+	—	—	.
C 24 B	+	—	—	.
K 128	+	—	—	.
K 129	+	—	—	.
K 131	—	+	—	.
TY 7	—	+	—	.
28 E	—	+	—	.
30 E	—	+	—	.
35 E	—	+	—	.
40 FE	—	+	—	.
40 HE	—	+	—	.
R 10 E	—	+	—	.
4051 CO	—	+	—	.
4211 CO	—	+	—	.
34497 CO	—	+	—	.
34498 CO	—	+	± *	.
41483 CO	—	+	—	.
41484 CO	—	+	—	.
4678	—	± *	+	.
Newson	—	—	—	.

Similar results by type-precipitin reaction with HCl and formamide extracts, slide and water-bath agglutination.

* Cross-reactions.

† Represented by strain Newson.

Type II. Fourteen strains (K 131, TY 7 and those labelled E and CO) fell into this type. They all absorbed the homologous antibodies from at least one serum and most of them from two of the three available sera.

This type was previously identified by Coffey (1942); she noticed that her six strains (strains CO) were members of the same type as strain K 131, which is confirmed here.

Some particular characteristics of the sera are worth considering. Serum 312 against 4211 CO, with the low end-titre of 1/320, was type-specific and gave comparable results with the different tests and extracts. Serum 308, prepared against K 131, varied in that the first bleeding was completely type-specific by the precipitin reaction, while the second bleeding showed cross-reactions with heterologous groups and a staphylococcus extract; however,

both bleedings were non-specific by slide-agglutination. By diluting the serum to titre by the dropping method, the cross-reactions could be avoided without the need of further absorptions. Reactions with the serum 227 against TY7 also gave interesting data: the sixth bleeding was type-specific, but the previous one reacted with A and G groups; however, in contrast to serum 308, it was not made type-specific by dilution to titre. This seems to be directly related to the agglutinin content because, whereas the successfully diluted serum 308 had a titre of 1/640, the present serum had a titre of only 1/160.

Type III. This type was formed by the single strain 4678. Its homologous antiserum 294 was found to be type-specific in the first three bleedings but showed group specificity in the fourth bleeding. However, sera of all these bleedings cross-reacted by slide agglutination with strains of type I. On account of low agglutinin content (1/80) dilution to titre did not eliminate the heterologous reactions, but the serum was rendered specific by heterologous absorption.

Type antigen characteristics of Streptococcus subacidus

Since at least the *Streptococcus subacidus* strains K128 and 34498CO fell into distinct serotypes of *S. infrequens*, it was at first concluded that no relationship was found between the biochemical and the antigenic differentiation of these two species. However, in assessing the type antigenic behaviour of the *S. subacidus* strains, evidence may be brought to prove that the two streptococci are antigenically dissimilar. Thus, the *S. subacidus* 4678 showed that, while forming a distinct type of its own (type III), it also reacted by precipitin and agglutinin tests with the three different sera prepared against strains of type II. This strain 4678 also totally exhausted all the antisera of their type II agglutinins. Tests were repeated several times, and it was observed that absorption was always complete with doses equal to the minimal absorbing doses of the homologous organisms. It seems possible, therefore, that *S. subacidus* possesses at least two type antigens of which only one is shared by *S. infrequens*. It is interesting to note that these findings agree with an early observation of Minett & Stableforth (1934), who mentioned that *S. infrequens* shows only one of the two antigens exhibited by the 'low acid streptococci', the designation given by them to *S. subacidus*.

Antigenic complex

Hirst & Lancefield (1939) have stated that cocci from group A could be extracted by hot HCl more than once. There is reason to believe that it will be so with other serological groups and experience has confirmed that all the twenty haemolytic strains examined here can be satisfactorily extracted at least three times. This procedure was followed in the hope that it might bring about some evidence of the nature and distribution of the different antigenic substances in the cells of the group here concerned.

Ethanol fractionation was applied to crude HCl extracts of seven strains from three different serotypes and against which homologous type sera were available (Table 3). It was observed that, while the protein precipitates of the

Table 3. *Precipitin (group and type) homologous reactions with antigenic fractions of haemolytic group E strains*

Antigens ...	Ethanol soluble C substance		Ethanol insoluble (protein) fraction											
	Group E serum		Type I				Type II				Type III			
	Serum 273	Serum 278	Serum 250	Serum 308	Serum 227	Serum 311	Serum 312	Serum 294				
Extractions	1	2	1	2	1	2	1	2	1	2	1	2	1	2
C24B	++	++	+	+	+	+	+	+	+	+	+	+	+	+
K129	++	++	+	+	+	+	+	+	+	+	+	+	+	+
K131	++	++	+	+	+	+	+	+	+	+	+	+	+	+
TY7	++	++	+	+	+	+	+	+	+	+	+	+	+	+
28E	++	++	+	+	+	+	+	+	+	+	+	+	+	+
4211 E	++	++	+	+	+	+	+	+	+	+	+	+	+	+
4678	++	++	+	+	+	+	+	+	+	+	+	+	+	+

Nos. 1 and 2 refer to first and second extractions.

second extractions were obtained carbohydrate-free after three ethanol washings, the precipitates of the first extractions only showed purity after the fourteenth ethanol treatment. This may mean that the amount of protein is greatest at the surface of the bacterial cells. The C-substance solutions obtained from the repeated extractions of all the haemolytic strains gave typical carbohydrate rings in the presence of group E antisera. As the reaction was consistently positive, it seems that the substance responsible for group-specificity is polysaccharide in nature. Most of these solutions of multiple extraction induced the formation of ring precipitates of similar intensity when geometrically diluted from 1/2 to 1/16, suggesting that the group substance exists not only at the surface but also on the subsurface portions of the cells. None of the protein fraction solutions gave any reaction with its homologous serum, and the conclusion may be drawn that the type-specific fraction was dissolved by the ethanol and is, like the group substance, of polysaccharide nature. Two factors may corroborate these findings: first, the parallelism found in the order of antibody appearance in the serum induced by the streptococci of group E and of group B which, as proved by Lancefield (1934, 1938) have also a polysaccharide as the type-specific substance. Secondly, it was possible to type strains of group E by using formamide extracts—solutions of polysaccharide—which react with sera deprived of group antibodies. Lancefield (1940-1) also stated that of groups other than A, B or C, less is known about the type-specific substances, but the evidence suggests that polysaccharides are chiefly concerned.

Preparation of antisera for serological group E

In the preparation of antiserum for group E, hyper-immunization must be avoided as group antibodies may appear soon after the first inoculations and before type-specific antibodies. This peculiarity is probably responsible for the difficulties experienced in the preparation of this antiserum. In fact, it happened that two samples of sera from different sources and labelled 'group E specific' proved to be only specific for serotype II. No substantial difference could be found between heat-killed and formolized immunizing suspensions.

During the attempts at serum absorption the selection of a satisfactory absorbing strain was one of the main difficulties encountered. As reported by Eisman (quoted by Krumwied, 1943) absorbing sediments could be used more than once if they were washed and heated at 100° after use. The absorbing power of the heterologous crossing strains varied considerably, but no difference was found between the use of heat-killed, formol-killed or live cocci. Group agglutinins were present in sera shown to be specific by the type-precipitin reaction, but these sera may in fact contain C antibodies before group antibody can be detected. Nucleoprotein P seems also to play a part in cross-agglutination, although, like the C substance, not consistently; thus, while one group E antiserum with P-antibodies showed group agglutination, another similar one proved to be completely type-specific, an observation supported by Lancefield (1940-1).

DISCUSSION

The confusion in the terminology of the organisms here concerned, especially *Streptococcus infrequens*, deserves some attention. The term *S. infrequens* was originated by Holman (1916) for a group of haemolytic strains of infrequent occurrence, isolated from human pathogenic conditions, fermenting lactose, mannitol and salicin. Brown, Frost & Shaw (1926) also described two strains of *S. infrequens* isolated from sporadic cases of human septicaemia and pneumonia respectively. The name *S. subacidus* was also used by Holman (1916) for those pathogenic strains which showed no action with the three sugars tested. Holman's contentions regarding the pathogenicity of these organisms were generally supported by contemporary workers.

Later, Frost, Gumm & Thomas (1927) applied these names to haemolytic strains isolated from milk which were biochemically similar to Holman's streptococci. The descriptions were thereafter generally used by authors, e.g. Sherman (1937), dealing with dairy bacteriology. In the light of the present knowledge, it is clear that no relationship exists between the human pathogenic strains and the haemolytic streptococci from milk which fall into group E.

So far, strains belonging to group E have not been reported in man, in health or disease. According to the work of several authors quoted by Pike & Fashena (1946), no streptococci of this group were found in over 25,000 swabs from men in many parts of the world. The investigations of Plummer (1941) in Canada and Gunnison *et al.* (1940) in America were similarly negative. The strain isolated by Coffey (private communication) from a suppurating wound in the hand of a dairy worker was probably an accidental association with an infection of staphylococcal origin, as large numbers of these organisms were also present. More detailed information is also necessary before a sound conclusion can be drawn regarding the aetiological relation of group E streptococci with pathological conditions in pigs (Stafseth & Clinton, 1941, Collier, 1951). It is reasonable to consider the haemolytic strains of group E as non-pathogenic, their normal habitat being milk (Topley & Wilson's *Principles*, 1946).

In order to re-adjust the term to its former pathogenic and human relationship, Brown (1939) presented *Streptococcus infrequens* as a member of group A, including in it the pyogenic strains which ferment lactose, mannitol, salicin and trehalose, but not sorbitol. For group E he proposed the name *S. lentus* to indicate that these organisms grow slowly and in minute colonies. Undoubtedly this unnecessarily confuses the issue. In the first place, slow growth is not a characteristic of group E streptococci; both stock and recently isolated strains have shown, in this respect, similarity with other streptococci. Thus, to avoid confusion by creating a new expression, it seems more reasonable to retain the designation for haemolytic milk strains of group E and to adhere to a principle already established.

Streptococcus infrequens is an organism of well-defined biochemical characteristics. The results of this work are similar to those recorded by most of the workers on this organism (Minett & Stableforth, 1934; Gunnison *et al.* 1940,

and Coffey, 1942). The characteristics given in *Bergey's Manual* (1948) for group E streptococci are also concordant, but they are presented together with those of *S. subacidus*. *S. subacidus* seems similar in all respects to the haemolytic 'low acid streptococcus' isolated from milk and described by Minett & Stableforth (1934).

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Substrates for *Myxococcus virescens* with Special Reference to Eubacterial Fractions

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SUMMARY: It was found that a mutant strain of *Myxococcus virescens*, able to grow dispersed in liquid media, oxidized the cytoplasmic lipids and proteins of *Escherichia coli*. Cell walls and nucleic acids of *E. coli* were not oxidized, and the acid soluble fraction was only slightly oxidized. It was also observed that the cytoplasmic contents of intact cells of *E. coli* were not available for oxidation. Glucose and starch were oxidized by the strain of *Myxococcus virescens* used.

Many of the fruiting terrestrial myxobacteria grow and produce fruiting bodies in the presence of other micro-organisms or in the presence of substances obtained from micro-organisms (Vahle, 1909; Pinoy, 1921; Solntseva, 1939; Oetker, 1953). The nature of the substrates for myxobacterial growth is not clear, however, although some indication may be inferred from the observations of Snieszko, McAllister & Hitchner (1943) that aqueous and saline extracts of disintegrated eubacterial cells proved superior to ethanol, acetone and ether extracts in the cultivation of myxobacteria. The present study, part of an investigation of the nutrition of *Myxococcus virescens* (Loebeck, 1954), was undertaken to determine what portion of the eubacterial cell is oxidized by this organism. Beebe (1941-2) and Singh (1947) observed that members of the genus *Myxococcus* are best isolated from enrichment cultures made with soil samples spread on bacterial streaks. In the present work, *Escherichia coli* was used as the substrate organism. Organisms were grown in media containing radioactive carbon, after which they were fractionated into various chemical classes and given to respiring myxobacteria. In other experiments, purified cell walls and cytoplasmic contents of *E. coli* were used as substrates for respiring myxobacteria. Because of the controversial data on the nutritive value of carbohydrates for these organisms (Stanier, 1942; Clark, 1954), uniformly labelled glucose and starch were also tested as substrates for oxidation by suspensions of myxobacteria. In each case, the respired carbon dioxide was collected and assayed for radioactivity.

METHODS

Organisms. The organism used in this study was a variant of *Myxococcus virescens*, M57. The mutant differed from the parent strain in that it was able to grow in a dispersed state when shaken on a rotary shaker. It had originally been obtained by repeated transfers of swarm cells from Tryptone agar to

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Tryptone broth. The mutant strain was still able to grow and fruit on bacterial streaks, but did not fruit in liquid cultures.

Escherichia coli, obtained from Dr E. J. Ordal, was the substrate organism.

Media. *Escherichia coli* was grown in a modification of the defined medium of Waring & Werkman (1942). Uniformly labelled ^{14}C -fructose, which was obtained from Tracerlab, Inc., Boston, Mass., was sterilized separately and added to the previously sterilized medium to a final concentration of 0.2 % (w/v). Litre flasks, containing 250 ml. of medium, were inoculated with the washings of a 24 hr. heart infusion slope culture, and shaken on a rotary shaker at approximately 25° until a good turbidity was obtained, usually 24 hr.

Myxococcus virescens was grown on 0.4 % (w/v) Difco Tryptone. Twenty ml. of a 24 hr. culture from this medium were used as the inoculum for litre flasks containing 250 ml. These flasks were shaken on a rotary shaker at approximately 25° .

Structural fractionation of labelled Escherichia coli. Purified cell walls were obtained by the method of Salton & Horne (1951), with slight modification. Cells of *E. coli* were disintegrated in water and the supernatant fluid containing the cytoplasmic constituents was retained for other experiments. The cell walls were then suspended in M-NaCl and subsequently washed with distilled water. Electron micrographs, taken at various stages in the purification of the cell walls, were used to determine whether the cell walls had been freed from whole organisms and cytoplasmic debris.

Biochemical fractionation of the cytoplasmic supernatant from disrupted Escherichia coli. The method of fractionation of Schneider (1945) was used. The trichloroacetic acid used for the precipitations was toxic to the myxobacteria and it was removed by evaporating each fraction to dryness over a steam bath.

Procedure for the oxidation of radioactive material by myxobacterial cells. Twenty-four to 32 hr. cultures of myxobacteria were harvested by centrifugation from the Tryptone medium, and washed twice with approximately 50 ml. tap water. Ordinary Warburg vessels were fitted with rubber vaccine-bottle tops and the centre wells filled with 0.2 ml. 19N-NaOH. The main compartment of each vessel received 2-3 ml. of freshly collected myxobacteria suspended in distilled water, usually about 10-25 mg. dry weight. The labelled fraction being tested was added to the side arm, and the side arm and the top were both sealed. The reactions of the myxobacterial suspensions and the labelled fractions at the time of addition were approximately neutral. The vessel was tipped and placed on a mechanical shaker at c. 25° . At the end of the shaking period, usually 5 hr., 0.3 ml. N-HCl was injected into the main compartment through the vaccine top to release bound carbon dioxide and the vessel was shaken 1 hr. more, after which the NaOH was removed from the centre well and the carbonate precipitated as barium carbonate with 0.5 ml. N-BaCl₂. The carbonate precipitate was filtered, washed, weighed and counted in a Tracerlab gas-flow counter.

Experiments with growing organisms were conducted in a similar manner, using larger Warburg vessels. These contained 25 ml. of 0.1 % (w/v) Tryptone in

the main compartment of the vessel. The radioactive sample was added to the side arm and the vessel contents sterilized by autoclaving at 120° for 20 min. 1 ml. 19N-NaOH was pipetted into the centre well, and the vessel inoculated with 10 ml. of a 48 hr. Tryptone broth culture of *Myxococcus virescens*. The cultures were grown on a mechanical shaker for 48 hr. or until a good turbidity was obtained, after which the respired CO₂ was precipitated from the centre well as before.

Radioactive carbon determinations. Carbon combustions on all samples to be assayed for radioactivity were performed by the wet-oxidation method of Van Slyke & Folch, using Barker's apparatus (Calvin, Heidelberg, Reid, Talbert & Yankowich, 1949).

Radioactive glucose and starch. These were prepared in our laboratory by Dr A. Markovitz, according to the method of Putman, Hassid, Krotkov & Barker (1948).

RESULTS

Manometric experiments with unlabelled substrates

In an effort to find suitable carbon sources for the organism, manometric studies were performed by conventional techniques of measurement of oxygen uptake in the Warburg respirometer at 31°.

The organisms for such experiments were grown in the following manner: 50 ml. flasks containing 12.5 ml. of 0.45 (w/v) Tryptone were inoculated with 7.0 ml. of 48 hr. culture from the same medium, and grown for 24 hr. at *c.* 25° on the rotary shaker. These cultures were used as the inoculum for 150 ml. of the same medium contained in 500 ml. flasks, which were shaken for an additional 24 hr. Organisms were harvested by centrifugation, and washed twice with tap water, after which they were suspended in *c.* 20 ml. tap water. 1 ml. of this suspension was used per Warburg vessel, and 1.0 ml. of Sorenson's 0.07M-phosphate buffer (pH 7.0) was also added to the main compartment of each vessel. Substrates were added in 0.2 ml. volumes to the side arm.

Table 1. *Oxygen uptake of resting Myxococcus virescens, M 57*

Substrate added	μ mole added/ vessel	O ₂ uptake (μ l./hr./ vessel)
None	—	47
Pyruvate	2.0	50
Glucose	0.4	45
Beta-alanine	2.0	43
Malate	20.0	35
Starch	2.0	45

The rate of oxygen uptake with glucose, pyruvate, beta-alanine, malate and starch as substrate is shown in Table 1. It is apparent that none of the substrates tested stimulated oxygen uptake. Heat-killed bacteria and milk, used in a similar experiment also failed to raise the rate of oxygen uptake. In other experiments, in an effort to reduce endogenous oxygen consumption, cell suspensions were shaken for varying periods of time, up to 14 hr., in tap

water or in phosphate buffer. Such measures, however, were not successful in lowering the high autorespiration.

Additional manometric experiments were performed to test the ability of the organisms to oxidize substrates, after having been grown in their presence. For this organisms were grown in Tryptone medium containing respectively 0.5 % (w/v) glucose, maltose or starch, and then tested for their endogenous rate and rate of oxidation of these substrates. The data, shown in Table 2, reveal the difference between endogenous rates and the others to be so slight that no significance can be attached to them.

Table 2. *Oxygen uptake by Myxococcus virescens, M 57, previously grown in the presence of glucose, maltose or starch*

Organisms grown on	Endog. rate	Oxygen uptake (μ l./hr./vessel)		
		Glucose (20 mg.)	Maltose (20 mg.)	Starch (20 mg.)
Glucose	72	71	n.t.	n.t.
Maltose	45	n.t.	60	n.t.
Starch	50	n.t.	n.t.	45

n.t. = not tested.

Oxidation of radioactive substrates

The experiments cited might be interpreted as indicating that either none of the substances tested serve as substrates for oxidation, or any one or all of them could be oxidized with a concomitant reduction in autorespiration. In an effort to test these possibilities, radioactive substrates were prepared (see under Methods), and given to respiring myxobacteria. The oxidation of these substrates by resting myxobacteria is shown in Tables 3 and 4. Data for growing cells are given in Table 5.

DISCUSSION

Early workers (Quehl, 1906; Vahle, 1909) were unable to enhance the growth of myxobacteria with glucose or starch. Imshenetskii (1941), working with a strain of *Sporocytophaga myxococcoides*, reported a toxic effect of glucose that was not due to the oxidation-reduction potential. Stanier (1942), using a strain of *S. myxococcoides* and two strains of *Cytophaga* sp., found that the toxicity was explained in part by the presence of toxic materials formed during heat sterilization. Clark's (1954) data, however, show an inhibition with filtered or heat-sterilized glucose. Solntseva (1941) reported the disappearance of 20.5 and 18.5 % glucose respectively from cultures of *Chondromyces* and *Melittangium* spp., and Mishustin (1942) reported the disappearance of 3 % of the products resulting from starch hydrolysis with a strain of *Chondrococcus* sp.

Our results show that unheated and autoclaved glucose were oxidized to the same extent, within the limit of error, i.e. 16 and 17 %, respectively. It would seem, however, that autoclaved glucose may be somewhat toxic to growing organisms, since growth in the control vessel without glucose gave an optical

Table 3. *Oxidation of labelled carbohydrates and structural fractions of Escherichia coli by resting Myxococcus virescens, M57*

Substrate	Period of incub. (hr.)	Myxo. in vessel (mg.)	Substrate added (mg.)	CO ₂ evolved (expressed as BaCO ₃)		Total c.p.m.* added	Total c.p.m. recovered as CO ₂	Substrate oxidized to completion (%)	Average (%)
				With substrate added (mg.)	Endog. control (mg.)				
Living intact <i>E. coli</i>	5½	10.8	1.6	6.0	5.3	1795	105†	5.9	5.1
	5½	10.8	3.2	6.3	5.2	3590	157†	4.3	
Cytoplasmic supernatant	5½	12.0	0.5	8.7	5.9	394	110	27.9	28.0
	5½	10.8	1.9	7.7	5.3	1860	570	30.6	
	5½	10.8	3.8	7.6	5.2	3720	955	25.6	
Cell walls	2	28.8	1.5	8.0	5.7	2725	18	0.6	3.5
	2	28.8	1.5	7.4	5.7	2725	18	0.6	
	5½	12.0	0.7	6.3	5.9	937	47	5.0	
	5½	12.0	0.7	7.0	5.9	937	56	6.0	
	5½	10.8	0.5	5.3	5.3	1201	58	4.8	
¹⁴ C-starch	5½	10.8	1.0	5.1	5.2	2403	93	3.9	23.1
	5	25.5	0.41	16.7	12.1	593	162	27.3	
	5	25.5	0.82	15.2	12.2	1187	317	26.6	
	5	25.5	1.23	15.9	12.9	1780	382	21.5	
¹⁴ C-glucose	5	25.5	1.72	16.1	—	2375	408	17.2	16.0
	5	25.5	t.q.‡	15.6	12.9	10,000	1910	19	
	5	25.5	t.q.‡	15.7	12.2	20,000	2650	13	

* c.p.m. = counts/minute.

† Since the *E. coli* used here were living, they undoubtedly contributed a portion of the label by their own respiration.

‡ t.q. = trace quantity.

Table 4. *Oxidation of labelled Escherichia coli fractions by resting Myxococcus virescens, M57*

Substrate	Period of incub. (hr.)	Myxo. in vessel (mg.)	Substrate added (mg.)	CO ₂ evolved (expressed as BaCO ₃)		Total c.p.m. added	Total c.p.m. recovered as CO ₂	Substrate oxidized to completion (%)	Average (%)
				With substrate added (mg.)	Endog. control (mg.)				
Lipid fraction	5	25.5	0.13	12.6	12.2	892	418	46.8	33.8
	5	25.5	0.33	13.5	12.9	2230	468	20.9	
	5	25.5	0.61	15.1	12.2	1235	155	12.5	
Acid soluble fraction	5	9.4	0.35	8.5	7.3	263	27	10.3	7.2
	5	9.4	0.70	9.6	7.2	536	50	9.3	
	5	16.4	0.45	9.3	7.5	584	29	5.0	
Nucleic acid fraction	5	16.4	0.90	9.2	7.6	1168	62	5.3	33.1
	5	25.5	0.90	13.2	12.5	1168	70	6.0	
	5	9.4	0.35	7.3	7.3	550	205	36.9	
Protein fraction	5	9.4	0.70	7.2	7.2	1100	270	24.5	33.1
	5	16.4	0.28	9.0	7.5	283	62	21.8	
	5	16.4	0.56	8.0	7.6	566	89	15.7	
	5	21.4	0.56	11.5	12.3	1414	470	33.6	33.1
	5	21.4	1.12	12.1	12.7	2828	920	32.6	
	5	25.5	0.56	12.5	12.5	1414	730	51.6	
	5	25.5	1.12	13.1	12.9	2828	1372	48.5	

Table 5. Oxidation of labelled substrates by growing *Myxococcus virescens*, M 57

Substrate	Period of incub. (hr.)	Optical density	CO ₂ evolved (expressed as BaCO ₃)			Total c.p.m. added	Total c.p.m. recovered in CO ₂	Substrate oxidized (%)	Average (%)
			Substrate added (mg.)	With substrate added (mg.)	Endog. control (mg.)				
¹⁴ C-glucose	60	0.122	t.q.*	38.1	—	2000	375	15.0	17.0
	60	0.080	t.q.	35.3	—	20,000	3165	19.0	—
	60	0.145	None	—	37.0	0	0	—	—
Cytoplasmic supernatant of <i>E. coli</i>	48	—	0.95	17.7	—	930	203	21.8	20.7
	48	—	1.9	17.4	—	1860	366	19.6	—
	48	—	None	—	20.8	0	0	—	—
Cell walls of <i>E. coli</i>	48	—	None	—	21.8	0	0	—	—
	48	—	0.7	23.3	—	937	77	8.2	—
	48	—	0.7	24.3	—	937	100	10.7	—
	48	—	None	—	20.4	0	0	—	—
	48	—	None	—	20.4	0	0	—	—
	48	—	0.5	16.6	—	1201	61	5.0	7.5
	48	—	1.0	18.8	—	2403	147	6.1	—
	48	—	None	—	20.8	0	0	—	—
	48	—	None	—	21.8	0	0	—	—
Acid soluble fraction	60	0.10	0.61	35.3	—	1235	25	2.1	1.8
	60	0.11	1.22	36.9	—	2470	40	1.6	—
	60	0.145	None	—	37.0	0	0	—	—
Lipid fraction	60	0.105	0.16	40.7	—	1115	255	22.8	25.7
	60	0.108	0.33	39.3	—	2230	570	25.6	—
	60	0.108	None	—	38.2	0	0	—	—
	60	0.108	None	—	38.2	0	0	—	—
Nucleic acid fraction	64	0.082	0.23	15.6	—	584	13	2.2	2.1
	64	0.075	0.45	16.3	—	1168	25	2.1	—
	64	0.090	None	—	14.4	0	0	—	—
Protein fraction	64	0.078	None	—	16.8	0	0	—	—
	64	0.105	0.5	36.2	—	1414	345	24.5	25.2
	64	0.115	1.1	36.6	—	2828	765	27.0	—
	64	0.108	None	—	38.2	0	0	—	—
	64	0.108	None	—	38.2	0	0	—	—

* t.q. = trace quantity.

density reading of 0.145, whereas the readings obtained from the vessel containing a small quantity of glucose were 0.122 and, for the larger quantity, 0.080.

Of paramount importance is the observation that, while the proteins and lipids of killed *Escherichia coli* were readily oxidized by *Myxococcus virescens*, the protoplasmic contents of intact living *Escherichia coli* were not available to the myxobacteria. The average percentage of labelled carbon appearing in the respired carbon dioxide, with living *E. coli* as a substrate, was 5.1 %. This represents a maximum figure since some, if not all, of this is derived from the endogenous respiration of *E. coli*.

The average value obtained for the complete oxidation of the purified cell walls of *Escherichia coli* by resting myxobacteria is 3.5 %. It should be emphasized that the cell-wall preparations may contain small amounts of some contaminating components which the myxobacteria are able to utilize. In this connexion, it is interesting to note that, judging from their appearance in the electron microscope, some cell-wall preparations were more easily washed than others, and that these fractions gave the lowest amount of labelled C in the respired CO₂. In the experiment with growing cells, the extent of oxidation of cell wall was only slightly higher and this may well be the result of hydrolysis of cell-wall material during autoclaving.

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Biophysical Studies of the Virus System of Vesicular Stomatitis

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SUMMARY: Biophysical studies of the virus system of vesicular stomatitis passaged in eggs showed that the major part of the infectivity was associated with a component of sedimentation coefficient 625*S*. A component of sedimentation coefficient 330*S* was observed also, and is probably a non-infective product of the disintegration of the 625*S* component. These components contribute about 35 % of the total complement-fixing activity of the virus system. All infective materials were handled in subdued light (Skinner & Bradish, 1954). Electron micrographs of concentrates of the infective fraction revealed rods, of length 175 $m\mu$. and diameter 69 $m\mu$., and almost spherical granules of diameter 65 $m\mu$. These particles are identified with the 625*S* and 330*S* sedimentation components. The remaining 65 % of the total complement-fixing activity was associated with two discrete components of sedimentation coefficients about 20*S* and 6*S*. The first of these components may contribute up to 0.1 % of the total infectivity of the virus system. The structure of the virus system is discussed in relation to the data obtained.

Ultracentrifugal studies of the virus system of foot-and-mouth disease (Bradish, Brooksby, Dillon & Norambuena, 1952) demonstrated that infectivity and complement-fixing activity were associated with discrete components of sedimentation coefficients 70*S* and 8*S*, respectively. These values correspond with equivalent particle diameters of about 20 and 7 $m\mu$., respectively. The extension of studies of this type to the virus system of vesicular stomatitis presents advantages because of the greater mass of the infective particle which, on the basis of earlier ultrafiltration and ultracentrifugation data (Galloway & Elford, 1933; Elford & Galloway, 1937) has an equivalent diameter of about 75 $m\mu$. This larger particle facilitates the use of certain biophysical methods which were not employed in the previous study (Bradish *et al.* 1952). Of particular value is the comparison which may be made between the data obtained by the biological assay of ultracentrifugal fractions and those derived by direct optical-analytical ultracentrifugation and by electron microscopy. The results obtained are presented in this paper.

METHODS

Handling in subdued light

It was observed by Skinner & Bradish (1954) that the infectivity of suspensions of many viruses, including those of the virus of vesicular stomatitis, was decreased significantly by exposure to light. Special precautions were taken

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therefore to minimize the possibility of errors arising from this cause. When infectivity was to be studied the successive operations, from the collection of the infective starting material to the inoculation of the dilutions of experimental fractions, were conducted in subdued light. A satisfactory lighting for this purpose was provided by one or two 15 W. red lamps placed at least 20 in. from the virus suspensions. This represents a maximum degree of illumination of about 0.3 foot-candle and compares with a degree of illumination of 200 to 1200 ft.c. at a typical laboratory bench.

The virus strains used and the preparation of virus suspensions

Vesicular stomatitis virus. Virus of both immunological types was used: strain Ind.C. of the Indiana type and strain NJ.M of the New Jersey type. These strains were maintained in developing eggs by passage by the chorioallantoic method (Beveridge & Burnet, 1946) at approximately weekly intervals. Before inoculation the eggs were incubated at 36° in a commercial forced-draught incubator. For the production of infective fluid, inocula of 10^4 ID₅₀ doses, containing 200 to 300 units penicillin, were dropped on to the chorioallantoic membranes. The embryos died within 18–24 hr. With virus of strain NJ.M eggs of 7-days incubation were used since in older eggs this strain did not always kill the embryos. For virus of strain Ind.C only 9- or 10-day eggs were used because of the greater yield of infective fluid. Inoculated eggs were incubated at 35° in an ordinary bacteriological incubator, and as soon as possible after the deaths of the embryos the allantoic and amniotic fluids were withdrawn. Fluids from each batch of eggs were pooled and stored at 4° for not more than 24 hr. before ultracentrifugal and other studies. Unless stated otherwise in the text, all data refer to experiments with pooled egg fluids infected with virus of strain Ind.C.

Estimation of infectivity of virus suspensions. Serial tenfold dilutions of the virus suspensions were prepared in a medium consisting of 0.15M-NaCl adjusted to pH 7.6 by the addition of phosphate salts to a total molarity of 0.005. This medium was used as the standard diluent throughout this study. Unless stated otherwise, 10 or 12 eggs were inoculated with each dilution of the series and were subsequently examined twice daily for 3 days. In titrations of the infectivity of suspensions of virus of strain NJ.M eggs of 7-days incubation were used; with suspensions of virus of strain Ind.C eggs of 7 or 8 days, or more rarely 9 or 10 days were used. The specificity of death of each embryo was determined by a complement-fixation test on a small sample of pooled allantoic and amniotic fluids from each egg. Samples from eggs classified as 'specifically dead' fixed 2M.H.D. of complement in the presence of excess of the homologous guinea-pig antiserum.

Calculation and presentation of infectivity data. The 50 % end-point dilutions were calculated by the method of Reed & Muench (1938) and only eggs classified as 'alive' or 'specifically dead' were included. For convenience of presentation infectivity titres are expressed as the logarithms to the base 10 of the reciprocals of the 50 % end-point dilutions. Thus a 50 % end-point dilution of

$10^{-6.0}$ is expressed as an infectivity titre of 6.0. An infectivity titre of <6.0 indicates an end-point dilution between 10^0 and $10^{-6.0}$.

Estimation of complement-fixing activity. Complement-fixing activity was estimated by the method described by Brooksby (1952). Serial twofold dilutions of the experimental sample were tested with excess of the specific antisera. In view of the observed greater lability of the antigenic material in vesicular stomatitis the accuracy of determination of complement-fixing activity in this work is about $\pm 15\%$ and is inferior to that observed in previous studies of the virus system of foot-and-mouth disease (Bradish *et al.* 1952).

Ultracentrifugation

Ultracentrifugation in angle rotors. The methods employed were generally as described by Bradish *et al.* (1952). The Model E and Model L Spinco Ultracentrifuges (manufactured by the Specialised Instrument Corporation, Belmont, California, U.S.A.) were used with a range of angle rotors designed to accommodate capped Lusteroid tubes of capacity from 8 ml. (Rotor C, 14 Tubes) to 100 ml. (Rotor L20, 10 tubes). Before fractionation all materials were clarified by centrifugation so that, ideally, about 10% of the heaviest specific component to be separated was deposited with the non-specific gross material. In most cases only the upper nine-tenths of the supernatant fluids were retained for use. This ensured that the clarified starting materials were as free as possible from the gross materials which otherwise would have appeared in subsequent deposits. This clarification procedure, appropriately adjusted to the sedimentation properties of different fractions, was employed throughout this study.

During ultracentrifugation in the refrigerated vacuum chambers the loaded rotors were maintained at about 5° . After ultracentrifugation the tubes were withdrawn carefully from the cold rotor in groups of three or four and placed in a rack indicating the levels required for sampling. Supernatants were withdrawn without delay by means of capillary pipettes and teats. The tip of each capillary pipette was bent to the form of a J and was held just below the meniscus in the tube as the supernatant was withdrawn slowly. After collection samples were stored at 4° until required.

In experiments where it was desired to recover the deposits from the centrifuge tubes the supernatant fluids were withdrawn completely by means of straight capillary pipettes. The tubes were allowed to drain for a few minutes and any residual supernatant removed as before. The compact deposit remaining near the base of each tube was resuspended in a third of the required volume of the standard diluent by agitation for about half a minute in the bore of a capillary pipette. The remaining diluent was used to rinse the emptied tubes at least twice. Resuspensions and tube washings were then pooled, shaken well by hand, and returned to storage at 4° . In some experiments the deposit resuspensions were agitated mechanically during storage overnight. Resuspensions were clarified the following morning. Only the upper nine-tenths of each clarification supernatant fluid was removed in order not to redisperse the lightly packed deposits.

In most experiments deposits were resuspended in volumes of diluent equal to one-tenth to one-twentieth of the volume initially loaded into the centrifuge tubes. Thus the concentration per cycle was about 10-fold or 20-fold and two cycles of sedimentation and clarification were required to obtain a concentration of about 400-fold. In experiments on the concentration of the infective components the initial volumes of the clarified starting materials of 250 ml. to 1000 ml. were reduced to 2 ml. or less by two or three cycles of sedimentation and clarification extending over 2 or 3 days. Thus from 3 to 5 days elapsed between the collection of the infective pooled egg fluids and the titration and analysis of the derived fractions. It was generally unnecessary to exceed a concentration factor of about 400 since a final volume of about 1 ml. of each concentrated suspension was required for subsequent studies.

Procedures with a swinging-cup rotor. In addition to the angle rotors described above a special swinging-cup rotor was employed (Bradish *et al.* 1952) in conjunction with a range of fittings designed to allow samples of from 2.5 to 0.05 ml. to be centrifuged in strictly radial tubes. The four duralumin cups of the swinging-cup rotor were of 12.7 mm. internal diameter and were used without inserts to receive 2.5 ml. each of the experimental samples. An anti-convection grid of 7 mm. depth consisting of seven stainless steel tubes of 3.6 mm. internal diameter packed hexagonally within a larger stainless steel tube of 12.7 mm. external diameter (obtained through the courtesy of Accles and Pollock, Ltd., Oldbury, Birmingham) was placed with fine forceps at the base of each loaded cup. Since the wall thickness of the steel tubes is only 0.23 mm., the grids do not modify significantly the desired radial sedimentation of suspended particles yet provide protection against the convections which may arise during manipulation. At the conclusion of ultracentrifugation the samples of supernatant fluid were withdrawn carefully with J-capillary pipettes down to the level of the upper surface of the grids.

When volumes of less than 2.5 ml. were to be centrifuged plastic inserts of Perspex or Fluon (polymethyl methacrylate and polytetrafluoroethylene, products of Imperial Chemical Industries, Ltd.) were used. These inserts are cylinders of 12.7 mm. external diameter, drilled and polished to form cylindrical cups of 3 mm. or 6.5 mm. internal diameter and 22 mm. depth. To facilitate the collection of supernatant fluids and deposits the ends of the cups were rounded and the external surfaces of the cylinders ringed at the standard meniscus level and at the standard supernatant sampling depth. On the external surface of each cylinder were two longitudinal grooves which permitted handling with forceps and allowed each insert to be held securely between vertical pins on the sampling stand. Thus a reproducible procedure was available for the ultracentrifugation of volumes as low as 0.05 ml. The sampling procedures employed were as described for the tubes of the angle rotors.

Control tests indicated that neither infectivity nor complement-fixing activity was influenced significantly by the storage of samples in plastic or duralumin cups for 24 hr. at 4°.

Calculation and presentation of ultracentrifugal data. It was shown in the previous paper (Bradish *et al.* 1952) that the process of centrifugation in radial

or inclined tubes may be represented to a first approximation by an equation of the form

$$SG = \left(K \log_{10} \frac{r}{r_0} \right) \left(1 - \frac{C}{C_0} \right),$$

where r_0 is the radius of rotation of the meniscus in the centrifuge tube, r the radius of rotation at the sampling depth and C_0 the initial concentration of the component of sedimentation coefficient S Svedberg units. C is the concentration of this component in the standard supernatant sample defined by r_0 and r following the centrifugation process G . The parameter G , defined in the previous paper, represents the integration of the square of the spin of the rotor over the period of centrifugation, including periods of acceleration and retardation. G values have been calculated directly from the duration of the centrifugation process, and the total number of rotor turns. For convenience G is expressed in terms of time in hours and spin in units of 10,000 rev./min. Thus centrifugation at a constant spin of 40,000 rev./min. for 2 hr. contributes 32 units to the overall value of G . These mixed practical units may be converted to reciprocal seconds by multiplication by the factor $4\pi^2 \times 10^8$.

The numerical constant K has the value 5833 when sedimentation occurs in radial tubes and 5833/2.6, or 2240, when sedimentation occurs in inclined tubes. The validity of this treatment of sedimentation in inclined tubes was discussed by Bradish *et al.* (1952). For any standardized sampling procedure the term $k = K \log_{10} r/r_0$ represents a constant which, once calculated from the dimensions of a rotor and its tubes, may be substituted in the equation $SG = k(1 - (C/C_0))$ and used to predict appropriate conditions for clarification and sedimentation or to estimate activity-average sedimentation constants from sampling and assay data. Characteristic values of the dimensionless constant k are given in Table 1 for the rotors and tubes employed. Thus, when a component of sedi-

Table 1. Values of the constant $k = SG / \left(1 - \frac{C}{C_0} \right)$, for various standard sampling procedures

Rotor	Inclination of tubes to vertical	Volume loaded per tube	Value of k for indicated sampling procedure		
			Upper $\frac{1}{4}$ super-natant withdrawn	Upper $\frac{3}{4}$ super-natant withdrawn	Total super-natant withdrawn
<i>C</i>	37°	8 ml.	150	450	600
<i>L40, D, L20</i>	26°, 18°, 18°	10, 25 to 36, 100 ml.	150	330	420
Swinging-cup	90°	2.5 ml. in duralumin cup with anti-convection grid	Supernatant withdrawn to upper surface of grid		420
		0.5 ml. in plastic insert	0.35 ml. super-natant withdrawn		335

mentation coefficient $100S$ is to be removed by centrifugation from the upper 6 ml. supernatant fluid in a C rotor tube, a G value of 4.5 units is required. This G value would be obtained by bringing the C rotor up to 20,000 rev./min.,

maintaining this spin for 1 hr., and then retarding the rotor at the standard rate. Alternatively, the same G value would be obtained if the C rotor were accelerated to 39,000 rev./min. and immediately retarded at the standard rate without any period at constant spin. This second condition enables the required G value to be attained in the shortest time.

Optical-analytical ultracentrifugation. The Spinco A and B analytical rotors were employed. The sample to be studied was loaded into the sector cell of appropriate length and centrifuged in the vacuum chamber without cooling by refrigeration. The rotor speed was chosen to allow at least five successive sedimentation diagrams to be photographed during centrifugation at constant speed for not less than 1 hr. The positions of sedimentation boundaries were measured from the plates by means of a travelling microscope and the radius of rotation (r) for each boundary was calculated from these in the usual manner. Sedimentation coefficients were calculated by the method of least squares from the paired values of $\log_{10} r$ and G . The optical-analytical sedimentation coefficients thus obtained are expressed in terms of water at 20° according to the theory and data presented by Svedberg & Pedersen (1940). A partial specific volume of 0.750 was assumed for all corrections, unless stated otherwise in the text. Sedimentation coefficients are expressed in Svedberg units (10^{-13} sec.) and designated by S .

Electron microscopy. Small drops of the concentrate to be studied were placed on formvar- or collodion-coated mounting grids. The concentrates were fixed either by the addition of one drop of 0.1 % osmium tetroxide solution to each drop of concentrate or by the exposure of the grid and drop to osmic vapour in a small sealed chamber. In both cases exposure continued for 30 min. before the drops were allowed to evaporate to dryness. Buffer salts were removed from the specimen films by the application and removal of small drops of distilled water. The dried specimen films were shadowed at an angle of $\tan^{-1}(\frac{1}{8})$ or $\tan^{-1}(\frac{1}{4})$ by a layer of about 8 Å. of gold-manganin or 20 Å. of chromium before examination in an R.C.A. EMU 2b electron microscope. This instrument was fitted with a compensated objective pole piece (Hillier & Ramberg, 1947). Electron optical magnification was determined by the use of Dow polystyrene latex of empirical mean diameter 2590 Å. (Backus & Williams, 1948; Scott, 1949). Samples of latex were kindly supplied by Dr V. E. Cosslett and by Dr R. C. Backus.

Electron micrographs were taken at magnifications from $\times 8,000$ to $\times 16,000$. The dimensions of images of characteristic particles were measured directly from the plates with a travelling microscope providing an optical magnification of $\times 10$. Subjective errors were reduced by the measurement of the dimensions of every discrete particle which appeared on the plates.

Estimation of nitrogen concentration. The nitrogen contents of the fractions studied were determined by the micro-Kjeldahl procedure of Ma & Zuazaga (1942), with the apparatus described by Markham (1942). Alternatively, colorimetric estimations based upon the Nessler procedure were employed.

RESULTS

Partition of the virus system

As in the study of the virus system of foot-and-mouth disease (Bradish *et al.* 1952) an attempt was made to distinguish, in the virus system of vesicular stomatitis, between the sedimentation properties of the components responsible for infectivity and complement-fixing activity. For this purpose samples of each of a number of clarified starting materials were subjected to centrifuga-

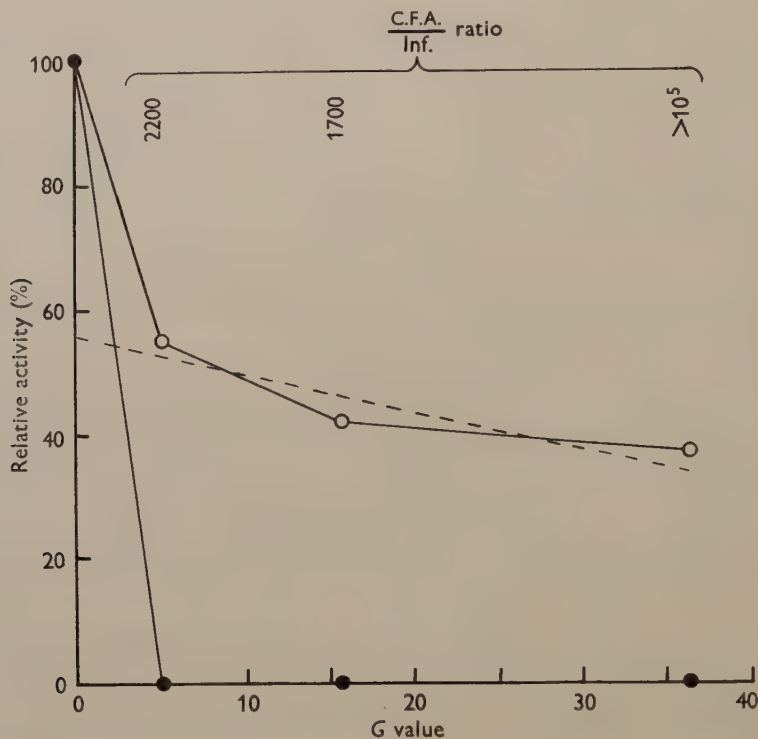


Fig. 1. Partition diagram showing decreases in activities of standard supernatant samples derived from a clarified starting material following ultracentrifugation in 0.5 ml. Perspex cups. ○ = complement-fixing activity; ● = infectivity.

tion at values of G ranging from 1 to 40 units. At the conclusion of each period of sedimentation, standard volumes of supernatant fluid were withdrawn from the centrifuge tubes and assayed for infectivity and complement-fixing activity.

These experiments were made either with the C rotor tubes, when standard 5 ml. volumes of supernatant fluids were withdrawn from the 8 ml. volumes loaded, or with the 6.5 mm. plastic inserts of the swinging-cup rotor when standard 0.35 ml. volumes of supernatant fluids were withdrawn from 0.5 ml. volumes loaded. The results of four typical experiments are shown in Figs. 1 and 2. In each case the complement-fixing activity fell to between 40 and 60 %

of its initial value in the supernatant fluid obtained at the lowest value of G , and thereafter fell much less rapidly in supernatant fluids obtained at greater values of G .

Similar results were obtained in experiments in which the clarified starting materials were subjected to several cycles of centrifugation at a fixed value of G . The standard supernatant withdrawn from the tubes of one cycle served as the starting material for the following cycle. Fig. 3 illustrates the partition of activity observed in these experiments.

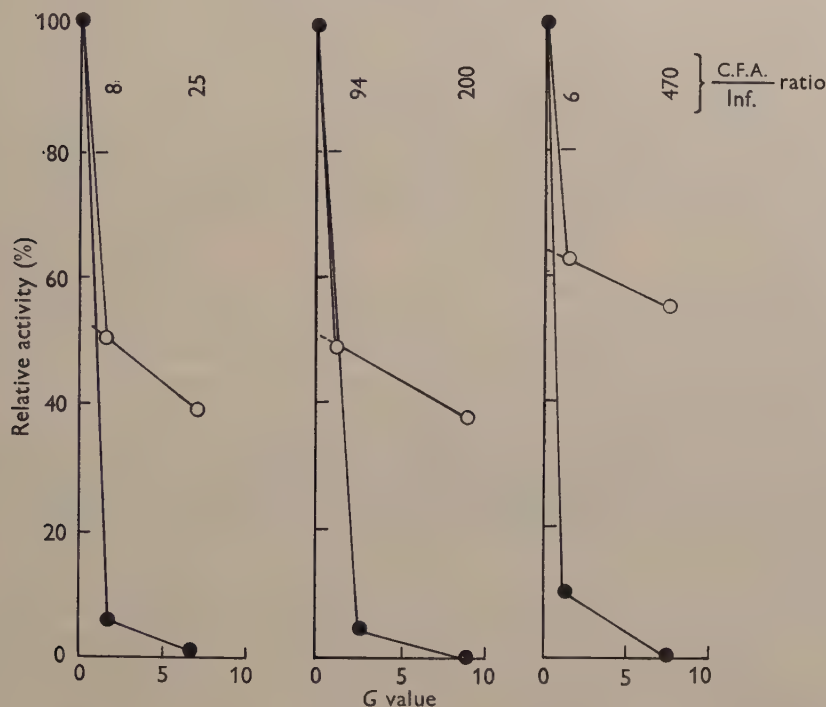


Fig. 2. Partition diagrams showing decreases in activities of standard supernatant samples derived from clarified starting materials following ultracentrifugation in 0.5 ml. Perspex cups. ○ = complement-fixing activity; ● = infectivity.

In all of these experiments the infectivity titres of the standard supernatant samples obtained after centrifugation at 6 units of G were about 3 logarithmic units lower than those of the clarified starting materials from which they were derived. It appeared likely that the discontinuity in the rate of fall of complement-fixing activity in supernatant samples arose from the presence of a rapidly sedimenting infective and complement-fixing component. The following experiments confirmed this view. Samples of supernatant fractions which retained about 50 % of the initial complement-fixing activity and less than 1 % of the initial infectivity were centrifuged at values of G ranging from 1 to 40 units. Standard supernatant samples were then withdrawn and assayed. The data obtained are shown in Fig. 4 and indicate that the complement-fixing

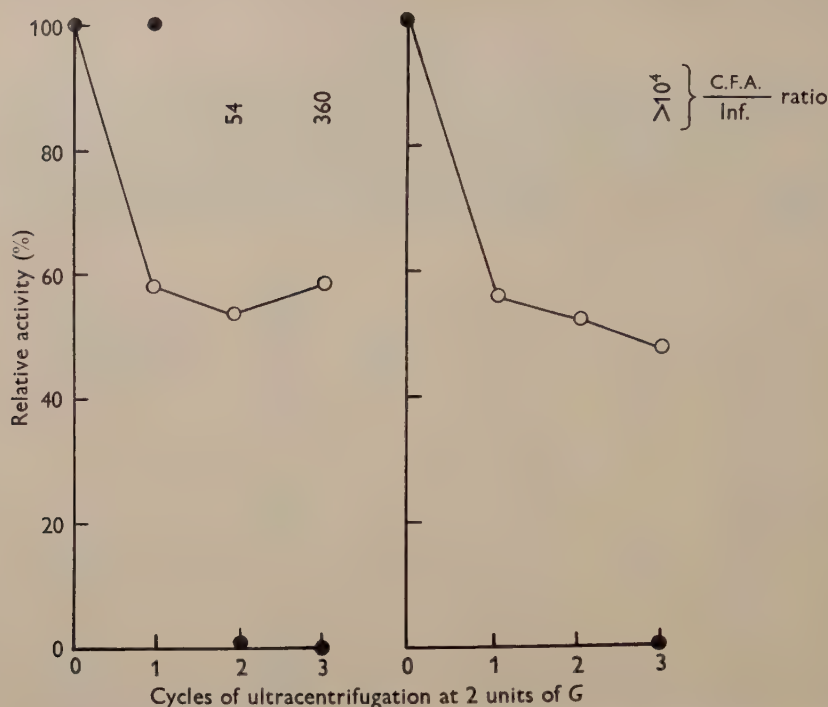


Fig. 3. Decreases in activities of standard supernatant samples derived from clarified starting materials following ultracentrifugation in inclined tubes of *C* or *D* rotors. \circ = complement-fixing activity; \bullet = infectivity.

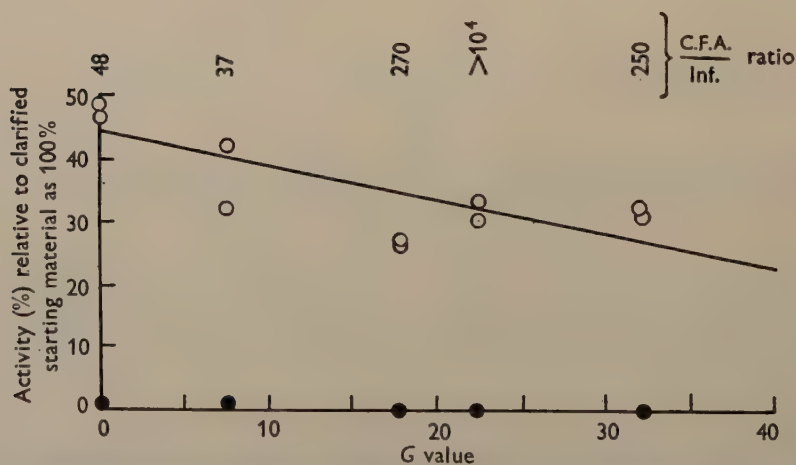


Fig. 4. Decrease in complement-fixing activity of standard supernatant samples derived from a complement-fixing fraction following ultracentrifugation in inclined tubes of *C* rotor. \circ = complement-fixing activity; \bullet = infectivity.

activity of supernatant samples fell very slowly with G and that the previous discontinuity at low values of G was absent.

The distribution of complement-fixing activity and infectivity in any fraction may be stated conveniently in terms of the ratio of complement-fixing activity to infectivity, both expressed as percentages of their initial values for the clarified starting material: this ratio is by definition unity for each clarified starting material. The value of this ratio for the first and second supernatant samples in the experiment of Fig. 1, for example, was about 2000. This implies that these samples of the slower sedimenting fraction retained about 65 % of the initial complement-fixing activity yet only 1 part in 3000, or 0.03 %, of the initial infectivity. Thus increases in the ratio of complement-fixing activity to infectivity (c.f.a./inf.) indicate the extent to which each cycle of ultracentrifugation removed the infective component from the complement-fixing fraction. The values of this ratio for the supernatant samples are given on the partition diagrams. That the removal of infectivity was not due to inactivation during manipulation is shown by the essentially quantitative recovery of infectivity in other parts of the same experiments. These data will be discussed later.

It was shown previously that data of the type shown in Figs. 1–3 provide the basis for the estimation of the percentage of the initial complement-fixing activity which is associated either with the larger infective component or with the smaller complement-fixing component. The appropriate extrapolation to the scale of activity is shown in these figures. In experiments of this type, extending over 2 years and with seventeen distinct samples of freshly collected pooled egg fluids, the percentage of the initial complement-fixing activity associated with the smaller complement-fixing fraction was within the range 55–80 % in fifteen cases and close to 20 % in the remaining two cases. Thus, in pooled egg fluids infected with Strain Ind.C of the virus of vesicular stomatitis, the rapidly sedimenting infective fraction is associated, in the majority of cases, with about 35 % of the total complement-fixing activity.

The complement-fixing component

Sedimentation properties. Experiments made to determine the sedimentation coefficient of the complement-fixing component are summarized in Table 2. The fractions analysed were obtained as standard supernatants from C rotor tubes following sedimentation of the infective components at 2 units of G . A complement-fixing component of sedimentation coefficient about $19S$ was observed in these experiments. However, in otherwise similar experiments in which the supernatant fractions analysed were obtained by two or three cycles of ultracentrifugation at higher values of G , a complement-fixing component of sedimentation coefficient about $6S$ was identified. A component of similarly low sedimentation coefficient was indicated by the analysis of data of the kind shown in Figs. 1 and 4. Thus, in the experiment of Fig. 1, the rate of fall of complement-fixing activity in standard supernatants was 0.61 % per unit of G . Substitution of this value in the differential form of the equation

$SG = k(1 - (C/C_0))$, putting $C_0 = 55\%$ and $k = 335$ appropriate to the sampling procedure, yields a sedimentation coefficient of $4S$. Again, in the experiment of Fig. 4, the rate of fall of complement-fixing activity in standard supernatants was 0.64% per unit of G . Then, putting $C_0 = 45\%$ and $k = 380$, a sedimentation coefficient of $5S$ is obtained.

Table 2. *Sedimentation coefficient of complement-fixing component in first-cycle supernatant fractions. Determinations in 2.5 ml. unlined duralumin cups of swinging-cup rotor.*

Starting material	G value	Radius of rotation (mm.)		C/C_0	Sedimentation coefficient (Svedberg units)
		Meniscus	Sampling level		
Allantoic fluid	10.3	72.0	88.5	0.69	14.5
Allantoic fluid	25.0	72.0	87.0	0.33	12.4
Allantoic mem-	15.0	72.0	87.0	0.29	21.5
brane suspension	15.1	72.0	87.0	0.34	20.0
Pooled egg fluids	20.4	72.0	86.5	0.44	23.0
Pooled egg fluids	22.8	72.0	86.5	0.43	20.6
Mean	—	—	—	—	18.7 ± 4.2

Equivalent values of the sampling constant k are: 520 for sampling level at 88.5 mm.; 480 for sampling level at 87.0 mm.; 465 for sampling level at 86.5 mm.

In view of this anomaly in the estimation of sedimentation coefficients experiments were made to test directly the possibility that the complement-fixing component was polydisperse and that the observed sedimentation coefficients of about $20S$ and $6S$ were actually activity-averages appropriate to the distribution of two or more components defined by the conditions of the preliminary fractionation.

Polydispersity of the complement-fixing component. A typical test of the degree of polydispersity was made as follows. A standard supernatant fraction was prepared from a clarified pool of egg fluids by deposition of the infective components at 2.5 units of G in the tubes of the $L20$ rotor. This first-cycle supernatant fluid was ultracentrifuged again at 100 units of G in the tubes of the C rotor and three supernatant pools were obtained representing the top third (A), middle third (B) and bottom third (C) of the total supernatant in each tube. The deposits, of about 3 mm. diameter, were resuspended in a third of the initial volume of standard diluent to provide, after clarification, a suspension (D) of the same volume as A, B and C. The sampling operations A, B, C and D were repeated following ultracentrifugation of the samples A, B, C and D at 100 units of G as before. The sixteen samples obtained (AA, AB, . . . down to DC, DD) were titrated for complement-fixing activity. The data obtained are shown in Table 3 and indicate a significant polydispersity of the complement-fixing fraction. A progressive decrease in the activity of the upper supernatant fluid was accompanied by an increase in the activity of the lower supernatant fluids and deposit resuspensions in the sample order: A, B, C, D. A single monodisperse component would have given rise to no differences between the distributions of activity in the second cycle groups

since the samples loaded (A, B, C, D) would have differed only in degrees of activity and not in sedimentation properties. The overall recovery of complement-fixing activity from the supernatant samples was satisfactory (80–110 %) but that from the deposit group (DA, DB, DC, DD) was low, probably through loss during clarification of part of the activity of the deposit suspension (DD).

Table 3. *A test of the polydispersity of the complement-fixing component*

Sample loaded for second cycle of ultracentrifugation	Complement-fixing activity with respect to starting material (%)	Complement-fixing activity of second cycle samples with respect to sample loaded (%)				Recovery of comple- ment-fixing activity with respect to sample loaded (%)
		A	B	C	D	
A	54	100	50	89	0	80
B	35	73	71	183	0	109
C	89	32	57	172	20	94
D	87	17	19	36	72	48

A further confirmation of polydispersity was obtained by the sedimentation analysis of the extreme samples AA and DD. The samples were analysed in pairs in the duralumin cups of the swinging-cup rotor (cf. Table 2) fitted with anti-convection grids. Following ultracentrifugation at 19 units of G the standard supernatants from the AA samples retained 86 % of initial activity while those from the DD samples were inactive. These data imply sedimentation coefficients of about $4S$ and at least $25S$, respectively. A parallel experiment in which AA and CC samples were analysed indicated 57 and 27 % retention of initial activity in the standard supernatant fluids, corresponding with sedimentation coefficients of $10S$ and $17S$, respectively. These typical data are consistent with the existence of two discrete complement-fixing components with sedimentation coefficients near $6S$ and $20S$, corresponding with equivalent particle diameters of 6 and $11\text{ m}\mu$, respectively.

Concentration and optical-sedimentation analysis of the complement-fixing components. Data presented in previous sections show that the complement-fixing fraction is associated with a complex of at least two components with sedimentation coefficients between 5 and 30 Svedberg units. Attempts were then made to concentrate these components in order to obtain more exact optical-sedimentation data. The concentration procedure was similar to that described for the infective fraction apart from the greater G values required for clarification and sedimentation. About 300 ml. of clarified starting material were clarified again in the tubes of the D rotor at a G value of 2 units. This deposited the infective fraction and yielded an upper supernatant pool consisting of the complement-fixing fraction. This fraction was centrifuged in the tubes of the D rotor at a G value of 45 units, sufficient to deposit components of sedimentation coefficient down to about $10S$. Deposits of about 5 mm. diameter were obtained and these were resuspended in 30 ml. of the standard diluent. The resuspension was clarified as before at 2 units of G and then

carried forward to a second and identical cycle of sedimentation and clarification. A faintly opalescent suspension of about 2 ml. volume was obtained which represented a 102-fold volume concentration and contained 0.06 mg.N/ml.

The complement-fixing activity of the concentrate, 1300 % of that of the starting material, was associated with an infectivity titre of 5.6 (starting material, 6.7), corresponding with a complement-fixing activity/infectivity ratio of 160. This value is less than that obtained for the unconcentrated complement-fixing fraction, but is much greater than the values of unity and about 0.3 for clarified starting materials and concentrates of the infective fraction. Thus the nature of the concentrate of the complement-fixing fraction is distinct from that of the infective fraction.

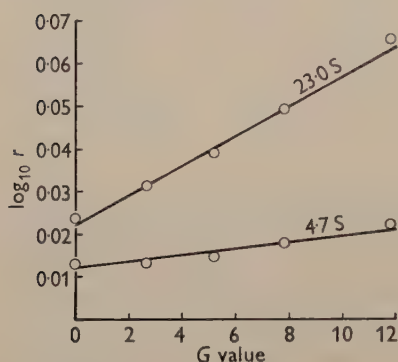


Fig 5

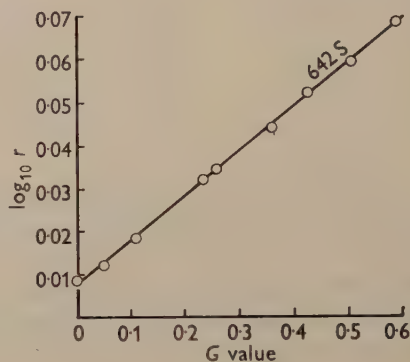


Fig. 6

Fig. 5. Sedimentation characteristics for components of concentrate of complement-fixing fraction. r = radius of rotation of sedimentation boundary; G = duration of centrifugation at constant speed expressed in standard integral form.

Fig. 6. Sedimentation characteristic for component of concentrate of infective fraction. r = radius of rotation of sedimentation boundary; G = duration of centrifugation at constant speed expressed in standard integral form.

The optical-sedimentation analysis of two separate concentrates of the complement-fixing fraction indicated components of sedimentation coefficients 21.5 (20.0 and 23.0) and about 6.0 (7.3 and 4.7) Svedberg units, respectively. The sedimentation diagram and characteristics for one of these concentrates are shown in Pl. 1, fig. 1, and Fig. 5. These experiments confirm the existence in complement-fixing fractions of at least two distinct components.

The major infective component

Separation and concentration. Evidence presented later indicates that the major part of the infectivity of suspensions of the virus system of vesicular stomatitis is associated with a component of sedimentation coefficient 625*S*. Before concentration of this major infective component the starting materials were clarified at a G value of 0.05 unit and the upper nine-tenths of the super-

natant fluids retained for use. Clarified starting materials thus obtained were centrifuged at a G value of 2 units which was sufficient to deposit a component of sedimentation coefficient 200–300 S , according to the rotor selected. In this first sedimentation only very small deposits were obtained; slight, white, opalescent deposits of 1–2 mm. diameter being obtained in C rotor tubes initially loaded with 8 ml. each of the clarified starting material. These deposits were dispersed readily in small volumes of the standard diluent and provided suspensions which remained turbid after clarification at a G value of 0.05 unit. Further cycles of concentration by sedimentation and clarification under these conditions yielded larger deposits and clarified resuspensions of marked turbidity. These concentrated suspensions of the major infective component, when viewed in normal daylight, appeared blue by scattered light and reddish brown by transmitted light, a behaviour which suggested the presence of particles of asymmetrical form. Typical assay data for these concentrates are presented in Table 4.

Table 4. *Concentration by ultracentrifugation of the major infective component in the virus system of vesicular stomatitis (strain Ind.C)*

Experiment number	27	31	36
Volume of clarified starting material (ml.)			500	250	1000
Infectivity titre of clarified starting material			7.2	6.8	7.5
Number of cycles of sedimentation and clarification			2	2	2
Concentration factor based on volume reduction			300	250	400
Infectivity titre of concentrate			9.3	8.7	9.2
Infectivity recovery in concentrate (%)			48	35	13
Complement-fixing activity of concentrate (%)			3100	1900	2000
Complement-fixing activity recovery in concentrate (%)			10	7	5
Ratio of complement-fixing activity to infectivity			0.2	0.2	0.4
Nitrogen content of concentrate (mg.N/ml.)			0.35	—	0.22

The observed infectivity recoveries of from 10 to 50 % suggest that a significant proportion of the infective component was lost during concentration. Probably the most severe loss occurred through the aggregation of the deposited material which was then either not resuspended or rejected during clarification. Also, even following efficient resuspension, aggregation of the infective component may influence the titration and result in a loss of infectivity. It may be noted that only about 10 % of the complement-fixing activity of the clarified starting material was recovered in the infective concentrate. The ratio of complement-fixing activity to infectivity in these concentrates was about 0.3 and is not significantly different from the unit value attributed to the clarified starting material. These values contrast with those of 1000 or more observed for the fractions associated with the complement-fixing activity.

The nitrogen content of concentrates varied from 0.03 to 0.4 mg.N/ml. corresponding with concentration factors based on volume reduction of from 100 to 400. These figures imply that the infectivity of the clarified starting materials was associated with a nitrogen content of about 0.0005 mg.N/ml., or only 0.1 % of the total initial nitrogen content.

Sedimentation data for the major infective component. A preliminary estimation of the sedimentation coefficient of the infective component in clarified starting materials was made by the capillary cell procedure described previously (Bradish *et al.* 1952). The data obtained are presented in Table 5 and indicate a mean minimum sedimentation coefficient of about 540 Svedberg units for the infective component. Further experiments of this kind were not made in view of the residue error already discussed (Bradish *et al.* 1952; Randrup, 1954), and in view of the difficulties of interpretation which arise as a result of the limited sensitivity of available titration procedures, particularly when more than one infective component may be present in the material to be analysed.

Table 5. *Capillary sedimentation data for the infective component in clarified starting materials*

Virus strain	Initial infectivity		C/C_0	Sedimentation coefficient (Svedberg units)
	titre	G value		
NJ.M	6.5	0.69	0.04	446
Ind.C	6.8	0.53	0.16	505
Ind.C	7.6	0.49	0.07	607
*Ind.C	7.5	0.44	0.13	630
Mean minimum sedimentation coefficient:				540

* A suspension of guinea-pig pad epithelium. Fifteenth passage in guinea-pigs. Same material as employed in experiment of Fig. 8.

Table 6. *Optical-analytical sedimentation data for components of the infective fraction*

Experiment number	Corrected sedimentation coefficient (Svedberg units)		Approximate nitrogen concentration of sample (mg.N/ml.)	Weighted mean sedimentation coefficient (Svedberg units)	
	Primary component	Secondary component		Primary component	Secondary component
23	597	311	0.30	580	307
27	562	298			
36	582	316			
21	615	321	0.10	622	322
34	681	333			
35	599	317			
16	670	—	0.05	625	—
16*	649	399			
18	692	—			
22	573	—			

* Concentrate analysed again following resuspension of deposit and storage overnight at 1°.

In further experiments, concentrates of the major infective fraction of the kind described in the previous section and in Table 4 were analysed in the optical cells of the ultracentrifuge. Over a period of 3 years nine concentrates were analysed in this way and yielded the sedimentation data presented in Table 6. Plate 2, fig. 4 shows a typical sedimentation diagram, and Fig. 6 the linear relationship between the calculation parameters $\log_{10} \tau$ and G .

In all concentrates a primary component, of sedimentation coefficient 560–680 Svedberg units, was predominantly responsible for the turbidity and optical properties of the suspensions. In seven of the nine analyses there also appeared a secondary component of sedimentation coefficient 300–400 Svedberg units which contributed from 5 to 20 % of the total sedimentable material.

The data obtained indicate a mean corrected sedimentation coefficient of 615 Svedberg units for the primary component and of 322 Svedberg units for the secondary component. Despite the extremely low concentration of specific material available for these analyses it is apparent from the data of Table 6 that much of the spread of the observed values arises from a dependence of sedimentation coefficient upon concentration. The extrapolation of these data to zero concentration indicates a sedimentation coefficient for each component which is about 10 Svedberg units higher than the mean value quoted above. Thus, for the purposes of further discussion, 625 and 330 Svedberg units will be adopted as the most probable sedimentation coefficients for the primary and secondary components in infinite dilutions of the infective fraction. The failure to observe any component of sedimentation coefficient greater than 625*S* and the remarkably close agreement between this value and the minimum value of 540*S* indicated by the capillary cell analyses of clarified starting materials (Table 5), justifies the conclusion that the component of sedimentation coefficient 625*S* is the major infective component in the virus system of vesicular stomatitis and that the 330*S* component is probably non-infective.

In one instance (Table 6) a concentrate of infective material was shown on first analysis to consist entirely of the primary 625*S* component. The deposit obtained from this analysis was resuspended in the water-clear supernatant fluid without removal from the cell and the characteristically turbid suspension obtained was analysed again after storage at +1° overnight. The suspension now contained about 10 % of the secondary 330*S* component in addition to the primary component. This observation suggests, as discussed later, that the 330*S* component is a degradation product of the predominant 625*S* component.

All of the above analyses were made in the standard diluent, 0.15*M*-NaCl adjusted to pH 7.6 by the addition of 0.005*M* phosphate salts. A preliminary analysis was made in a saline solution of greater density in order to obtain an estimate of the partial specific volume; data from this experiment are shown in Table 7. The calculated partial specific volume is close to 0.850 for both components and corresponds with a particle density of 1.18. These values must be regarded as provisional in view of the limited data and the uncertain influence of hydration. A partial specific volume of 0.850 was adopted for the correction of the sedimentation data presented in Table 6. Correction in terms of the 'average protein' partial specific volume of 0.750 would lower all quoted sedimentation coefficients by 1.72 %.

Estimation from sedimentation data of the particle dimensions of components of the infective fraction. The calculation of the dimensions of a particle requires, in addition to the sedimentation data, some knowledge of the shape of the

particle. This additional information was provided in the present study by the electron microscopy of concentrates of the infective fraction; this demonstrated the existence in these materials of rods, of axial ratio 2.55, and of almost spherical granules of axial ratio close to unity (see later). The inclusion of these

Table 7. *Estimation of partial specific volumes of components of a concentrate of the major infective fraction*

Medium	0.87 g. NaCl/100 ml.	21.98 g. NaCl/100 ml.
Sedimentation coefficient in medium at 20°		
Primary component	566.7	84.6
Secondary component	295.8	38.0
Correction factors at 20°		
ρ/ρ_0	1.0044	1.142
η/η_0	1.014	1.51
Calculated partial specific volume		
Primary component	0.847	
Secondary component	0.851	

axial ratios derived by electron microscopy in a calculation of particle dimensions from sedimentation data is justified since these ratios are independent of absolute dimensions and therefore require no knowledge of magnification factors. Thus particle dimensions obtained by the present calculations may be compared with those obtained entirely by electron microscopy, in order to assess the validity of the procedures employed.

Table 8. *Estimation from sedimentation data of particle dimensions of components of the infective fraction*

Identification	First		Second	
	625 <i>S</i>	330 <i>S</i>	625 <i>S</i>	330 <i>S</i>
Component	625 <i>S</i>	330 <i>S</i>	625 <i>S</i>	330 <i>S</i>
Shape of particle	Rod	Sphere	Sphere	Rod
Axial ratio	2.55	1.00	1.00	2.55
Frictional ratio	1.082	1.00	1.00	1.082
Diameter of sphere or equivalent sphere (m μ .)	83	58	79	60
Length of rod (m μ .)	155	—	—	113
Diameter of rod (m μ .)	61	—	—	44

Table 8 summarizes the data required for the calculation, and the particle dimensions derived. The calculation adopts a partial specific volume of 0.850 for both components and treats the rod as a prolate spheroid. Table 8 presents the two possible identifications: the first regards the primary 625*S* component as the rod and the secondary 330*S* component as the sphere; the second identification reverses this. The particle dimensions obtained on the basis of the first identification are consistent with those obtained by electron microscopy, and it is reasonable therefore to interpret the primary 625*S* component as a rod of length 155 m μ ., and diameter 61 m μ .. The secondary 330*S* component is interpreted as an almost spherical granule of diameter 58 m μ ..

Ultrafiltration studies by Galloway & Elford (1933) indicated a particle size

of 70–100 $m\mu$. for the infective component in guinea-pig vesicular fluid. Later ultracentrifugation studies by Elford & Galloway (1937) by the inverted-capillary tube technique indicated a particle diameter of 74 $m\mu$. on the assumption of a particle density of 1.20. These particle diameters are in excellent agreement with the diameters of the equivalent spheres, 79–83 $m\mu$., obtained in the present study for the 625*S* infective component in infective egg fluids (cf. Table 8).

Electron microscopy of concentrates of the infective component. During this work twelve separate concentrates of the infective component (cf. Table 4) were studied in the electron microscope. Micrographs were obtained within 2 or 3 days of the final concentration procedure, and in most cases at least two fields containing from thirty to sixty discrete particles were measured in detail in each case. Typical data for the dimensions obtained are given in Table 9; typical particle-size distributions are shown in Fig. 7, and typical

Table 9. *Typical data for the dimensions of characteristic particles in electron micrographs of concentrates of the infective component*

The figures in brackets show the number of discrete particles measured.

Source of infective material	Virus strain	Shadowing metal	Rod			Spherical granule diameter ($m\mu$.)
			Length ($m\mu$.)	Diameter ($m\mu$.)	Axial ratio	
Saline suspension of allantoic membranes	NJ.M	Gold-manganin	175 ± 25 (10)	66 ± 7	2.65	67 ± 9 (15)
Bovine tongue epithelium, 6th bovine passage	Ind.C.	Gold-manganin	193 ± 19 (5)	77 ± 8	2.50	71 ± 7 (175)
Allantoic fluid	NJ.M	Gold-manganin	173 ± 16 (27)	67 ± 10	2.57	.
Pooled egg fluids	Ind.C	Gold-manganin	180 ± 16 (39)	71 ± 3	2.55	.
Pooled egg fluids	Ind.C	Gold-manganin	170 ± 19 (55)	61 ± 12	2.80	.
Pooled egg fluids	Ind.C	Gold-manganin	176 ± 23 (56)	71 ± 10	2.46	59 ± 13 (182)
Pooled egg fluids	Ind.C	Chromium	164 ± 29 (50)	70 ± 15	2.32	69 ± 16 (261)
Pooled egg fluids	Ind.C	None	138 ± 26 (50)	59 ± 17	2.34	54 ± 9 (447)
Overall means	.	.	175 $m\mu$.	69 $m\mu$.	2.55	65 $m\mu$.

micrographs in Pl. 2. The rod, of mean axial ratio 2.55, was regularly observed and is regarded as the characteristic particle to be associated with the major infective component. The rod was absent from micrographs of control concentrates of normal pooled egg fluids and from micrographs of concentrates of the complement-fixing fraction.

In some micrographs the rods were accompanied by almost spherical granules of about the same diameter but of much greater contrast. The observation of this characteristic granule in electron micrographs of otherwise typical concentrates of the infective fraction suggests that it may arise from the breakdown of the primary rod. This view is consistent with the correspondence between the diameters of the rods and granules and with the existence of terminal granules of relatively greater contrast in the images of many rods (cf. Pl. 2). The observation of the secondary sedimentation boundary in the

optical-ultracentrifugal analyses of these infective concentrates supports the same conclusion.

The average dimensions of the rod, $175 \times 69 \text{ m}\mu$., and of the granule, $65 \text{ m}\mu$., obtained from electron micrographs compare very favourably with the dimensions based on sedimentation data (cf. Table 8) of $155 \times 61 \text{ m}\mu$. for the rod and $58 \text{ m}\mu$. for the granule. Differences between the dimensions obtained by the two methods are not significant in view of the standard deviations quoted in Table 9. Drying and shadowing artefacts in electron microscopy, and assumptions with regard to shape, hydration and density in sedimentation analyses, must all contribute to the variations and differences observed. Final estimates based on both methods for the average dimensions of the characteristic particles in concentrates of the infective fraction are: length of rod, $165 \text{ m}\mu$.; diameter of rod, $65 \text{ m}\mu$.; diameter of granule, $62 \text{ m}\mu$. These mean values are probably correct to within $\pm 10\%$.

Micrographs of concentrates of the infective fraction frequently showed rods in which about four transverse striations or turns of a spiral were identified. In addition, many rods exhibited at one end single trailing filaments of about $15 \text{ m}\mu$. diameter. Isolated filaments, frequently beaded, were also present. Granules and fragments of rods were observed in pairs lying side by side. Examples of such structures are circled in the micrographs of Pl. 2. These features are consistent with an interpretation of the structure of the intact rod as a coiled spring or platelet stack, capped by a single terminal granule. The evident fragility of the characteristic rod with respect to the methods of specimen preparation may account for the observed spreads of lengths and axial ratios (cf. Fig. 7). The possibility, however, that the data presented for the dimensions of the characteristic rod relate only to the most frequent fragment and that the intact rod is of greater length and rarely encountered under the present conditions, seems unlikely in view of the close correspondence between the data obtained by sedimentation analysis and from electron micrographs.

Micrographs of supernatant fractions associated with complement-fixing activity showed no characteristic features which could be distinguished with certainty from the non-specific content of the specimens. Many spherical particles of diameter $10\text{--}15 \text{ m}\mu$. were observed but these, although not inconsistent with the $20S$ component identified by sedimentation analysis, have not been related specifically to complement-fixing activity.

During the preparation of this paper, Chow, Chow & Hanson (1954) published an account of the morphology of vesicular stomatitis virus as revealed by the electron microscope. These authors, using an egg-passaged virus of the New Jersey strain, found rod-shaped particles of average length $210 \text{ m}\mu$. and diameter $60 \text{ m}\mu$. They also reported that 'the hair-like projections barely visible at the terminal ends and the slight subterminal enlargement may be the result of dehydration during preparation'. These findings are not inconsistent with the data presented in this section.

The minor infective component. It has thus been shown that the major part of the infectivity of suspensions of the virus system of vesicular stomatitis is associated with a rapidly sedimenting component of sedimentation coefficient

625*S*. For many further experiments, particularly following the recognition of the complexity of the relatively slowly sedimenting complement-fixing component, it was required to obtain supernatant fractions of maximum complement-fixing activity yet minimum infectivity. To provide such fractions clarified starting materials were ultracentrifuged in the inclined tubes of the *C* rotor at *G* values of from 2 to 4 units. The resulting standard supernatant

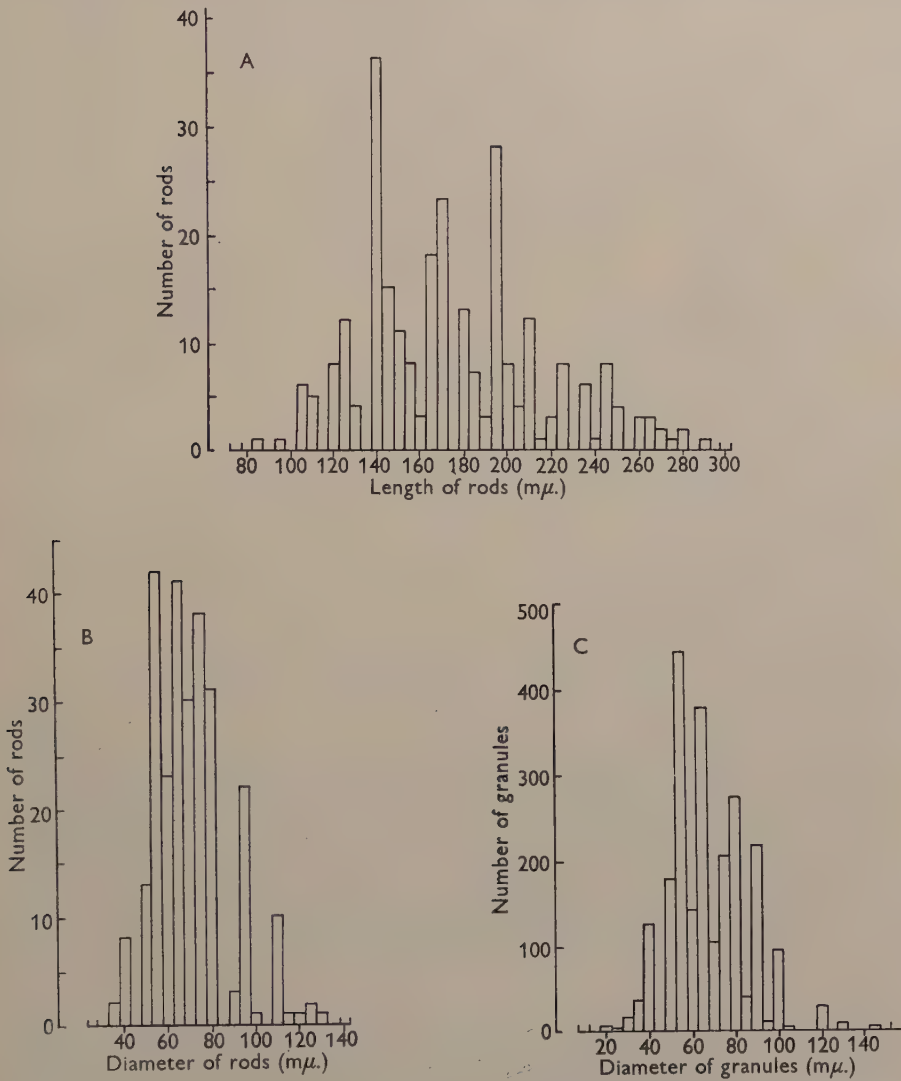


Fig. 7. A: distribution of lengths for 270 rods in micrographs of concentrates of the infective fraction. Average length of rods, 176 mμ. These data relate to five consecutive experiments which include the last three experiments of Table 9. B: distribution of diameters for 270 rods in A; average diameter of rods, 71 mμ. C: distribution of diameters for 2300 granules in micrographs of concentrates of the infective fraction; average diameter of granules, 68 mμ.

fluids were carried forward immediately for second cycles of ultracentrifugation at the same G values, and so on up to the 3rd or 4th cycle of ultracentrifugation. Alternatively, the G values were increased geometrically at each cycle up to 30 or 40 units of G at the 4th cycle. The starting materials and successive standard supernatant fluids were titrated for infectivity and complement-fixing activity. Typical results for egg and guinea-pig materials are shown in Fig. 8. It is apparent that although the infectivity titres fell rapidly by 2 or 3

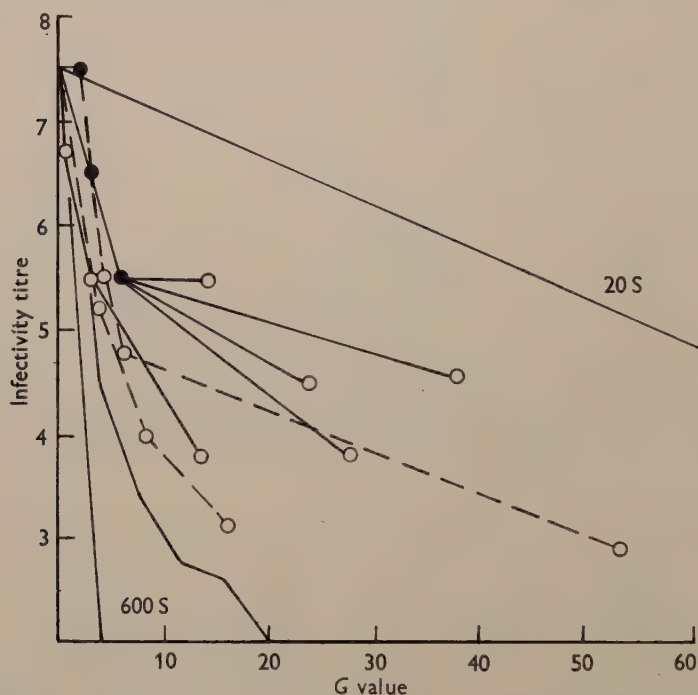


Fig. 8. Decreases in infectivity of standard supernatant samples derived from clarified starting materials following several cycles of ultracentrifugation. ● = inclined tubes of D rotor, 12.5 ml. withdrawn from 25 ml. loaded. ○ = inclined tubes of C rotor, 5 ml. withdrawn from 8 ml. loaded. An ultracentrifugation of, say, three cycles at 4 units of G is indicated by points at 0, 4, 8, 12 on the horizontal scale of G . The straight lines indicate the behaviour of ideal monodisperse components of sedimentation coefficients 600 S and 20 S . — = suspension of guinea-pig pad epithelium, 15th passage of virus of strain Ind.C. See Table 5 for additional data. All other starting materials were pooled egg fluids infected with virus of strain Ind.C.

logarithmic units in the early cycles the remaining infectivity was removed much less readily. Thus, even after 3 or 4 cycles of ultracentrifugation under conditions appropriate to the deposition of the major infective component, the supernatant samples retained up to 1 part in 1000 of the initial infectivity. The rates of decrease of infectivity during the later cycles of ultracentrifugation (cf. Fig. 8) and the association of the residual infectivity with the complement-fixing component already characterized, suggested the presence of the minor infective component of sedimentation coefficient about 20 S .

In additional experiments with radial tubes in the swinging-cup rotor, the third- or fourth-cycle supernatant fractions obtained above were loaded into the duralumin cups with anti-convection grids ($k=420$) or, for the third experiment, into the 0.5 ml. plastic inserts ($k=335$). In four separate experiments at 17, 20, 20, 14 units of G , respectively, the infectivity titres of the standard supernatant samples were 0.7, 1.7, 0.7, 0.7 logarithmic units lower than those of the fractions loaded. The starting material for the first experiment was a suspension of guinea-pig pad epithelium obtained at the 15th passage in guinea-pigs of virus of strain Ind.C. These data imply a minimum sedimentation coefficient of about 20S for the minor infective component.

The results of these preliminary experiments are consistent with the existence in the virus system of vesicular stomatitis of a minor infective component, of sedimentation coefficient about 20S, which contributes up to 1 part in 1000 of the initial infectivity of the starting material. This component may be associated with complement-fixing activity and may be identical with the 20S complement-fixing component.

*The influence of a number of factors upon the assay and stability
of infectivity and complement-fixing activity*

Exposure to light. The influence of exposure to light upon the infectivity of the unfractionated virus system was reported by Skinner & Bradish (1954). In additional experiments second-cycle concentrates of the major infective component (cf. Table 4) were exposed in McCartney bottles at 4° to a daylight intensity of about 250 ft.c. In one experiment samples of a dilution of a typical concentrate were exposed to daylight for 0.75 and for 5 hr. A third sample was stored in the dark at 4° for 5 hr. The observed infectivity titres were 3.5, 2.0 and 7.2, respectively. In a second experiment one sample was exposed to light for 1 hr. while the second was stored in the dark at 4° for 1 hr. The observed infectivity titres were 5.7 and 7.4, respectively. The minimum rate of decrease of infectivity observed in these experiments, 1.7 logarithmic units/hr., emphasizes the importance of the precautions adopted in this work to minimize exposure to light.

Complement-fixing activity is considerably less sensitive than infectivity to inactivation by exposure to light. Two unfractionated starting materials and two second-cycle supernatant fractions of the complement-fixing component were exposed at 4° to an artificial light intensity of 3000 ft.c. for 1–3 hr. A decrease of complement-fixing activity of only $20 \pm 7\%$ was observed in these four experiments. Thus exposure to light under normal experimental conditions was without significant influence on the complement-fixing activity of the fractions studied.

Storage in the dark. Clarified starting materials, second-cycle upper supernatant fractions of the complement-fixing component, and second-cycle concentrates of the infective component were stored in the dark for 1 and 24 hr. at 4, 20 and 37°. In no case did the observed change in the degree of complement-fixing activity exceed the $\pm 15\%$ accuracy of the test. Thus the

complement-fixing activity of the fractions studied was not modified significantly by storage for short periods at temperatures up to 37°.

Exposure to 56° for 1 hr. The exposure of the samples listed in the previous paragraph to 56° for 1 hr. regularly decreased the complement-fixing activity to 10 % or less of the initial level. The corresponding components in the virus system of foot-and-mouth disease, when treated in this manner, retained at least 70 % of their initial complement-fixing activity, indicating a significantly greater resistance to inactivation by heating.

Titration of infectivity in eggs and guinea-pigs. During the course of this work four clarified starting materials, two third-cycle concentrates of the infective component and two upper supernatant fractions of the complement-fixing component were titrated in parallel in eggs (9 per dilution) and guinea-pigs (10 per dilution). In these groups the 'egg' titres exceeded the 'guinea-pig' titres by 3.7 ± 0.2 , 4.0 ± 1.0 and 3.6 ± 0.3 logarithmic units, respectively. These data indicate a constant difference of about four logarithmic units between the infectivity titres indicated by the two hosts. The nature of the fraction inoculated was without apparent influence. Thus, on a relative scale of infectivity, these hosts would provide identical results in any study of the present kind.

DISCUSSION

The data presented in this paper demonstrate the complexity of the virus system of vesicular stomatitis as observed in infective egg fluids. The four components identified, of sedimentation coefficients 625*S*, 330*S*, *c.* 20*S* and *c.* 6*S*, may originate in two ways. It may be considered either that these components are distinct and separate products of the process of virus multiplication or that they arise from the disintegration of a single infective component. These interpretations are not mutually exclusive and either could result in similar biophysical findings. The data presented support the view that the components identified are representative of those which exist in egg fluids infected with virus of strain Ind.C.

The 625*S* component, identified as the major infective component, exists as a rod of dimensions 165×65 m μ . and is associated with about 35 % of the initial complement-fixing activity. The 20*S* component is probably also infective but does not contribute more than about 0.1 % of the initial infectivity. The 330*S* component, a compact spherical granule of diameter 62 m μ ., probably arises from the disintegration of the rod and may not be infective. The few experiments made with egg fluids infected with virus of strain N.J.M. and with guinea-pig- or bovine-passaged virus of strain Ind.C confirm these findings and do not reveal any host- or strain-variations.

The structure of the infective rod suggested by the electron micrographs, a coiled beaded filament or a platelet stack capped by an almost spherical granule of about the same diameter, is consistent with the observed sedimentation properties. Thus the 20*S* and 6*S* complement-fixing components may be fragments produced by the disruption of this filament. The implications of these components and possible structures in relation to chemical composition and

the mechanism of virus multiplication and decay cannot be discussed further until the kinetics of the interaction between the host cell and the complex virus system have been more fully investigated.

The multiplicity of components in the virus system of vesicular stomatitis as observed in infective egg fluids, together with the complexity of structure indicated by electron microscopy, demand further study of the relationship between physico-chemical structure and biological function. Of particular interest is the problem of the significance in the infective process of the smaller components in the virus system of vesicular stomatitis and foot-and-mouth disease, and possibly also of other virus diseases. By present techniques the study of components which may contribute only a small part of the total infectivity of a virus system is complicated by the contamination of fractions by residues of the major infective components. Despite these limitations, the association of trace infectivity with the 20S component in the virus system of vesicular stomatitis is indicated strongly by the present observations.

In this paper the complement-fixing fraction has not been referred to as the 'soluble antigen' but has been defined directly in terms of its sedimentation properties as the 20S and 6S components. These sedimentation coefficients correspond with equivalent particle diameters of about 11 and 6 m μ ., respectively. Both the present study and an earlier study of the virus system of foot-and-mouth disease (Bradish *et al.* 1952) indicate the presence of components which are significantly smaller than the basic subunit of about 12 m μ . diameter required by Polsen's postulate (1953).

It is a pleasure to thank Miss N. Smart and Dr R. C. Backus for their collaboration during part of this work. The valuable criticism and advice given by Dr I. A. Galloway, the Director of this Institute, is acknowledged with gratitude. Our grateful thanks are also given to Mr E. Scoates, for undertaking many of the complement-fixation tests, and to Misses H. Wigman and C. Worner and Messrs T. Burrows, J. German, J. Kirkham, P. Mitchell and A. Wells for other competent assistance.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Sedimentation diagrams for components of concentrate of complement-fixing fraction. Nitrogen content of concentrate, 0.06 mg.N/ml.; cell length=30 mm., $\theta=35^\circ$. Exposures taken 20, 30, 40 min. after attainment of 40,000 rev./min. Corrected sedimentation coefficients 20.0S and 7.3S.

Fig. 2. Sedimentation diagrams for components of concentrate of infective fraction. Nitrogen content of concentrate, 0.10 mg.N/ml.; cell length=30 mm., $\theta=20, 15, 15, 15^\circ$. Exposures taken 20, 30, 40, 50 min. after attainment of 7000 rev./min. Corrected sedimentation coefficients 599S and 317S.

PLATE 2

Electron micrographs of concentrates of the infective fraction derived from pooled egg fluids infected with virus strain Ind.C. Fig. 3: fivefold dilution of third-cycle resuspended deposit. Fig. 4: undiluted second-cycle resuspended deposit, see fifth entry in Table 9. Fig. 5: tenfold dilution of third-cycle resuspended deposit as used in Fig. 3. Figs. 3 and 4 show typical rods; Fig. 5 shows granules with some rods. Other features are indicated as follows; *R*=rods with dense terminal granules; *S*=rods showing cross bands or striations; *F*=trailing or isolated filaments; *RG*=rods or rod fragments associated with detached granules.

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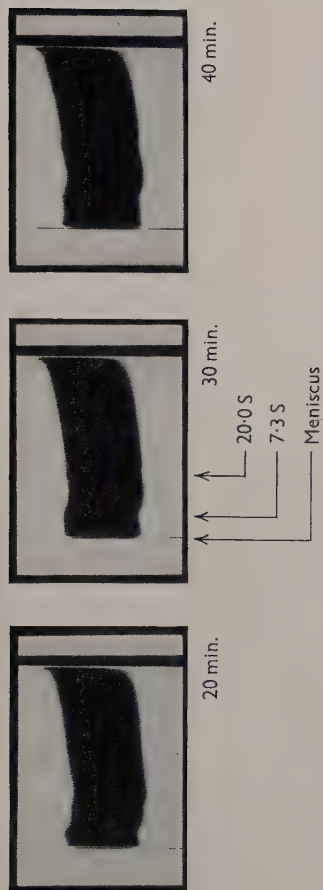


Fig. 1

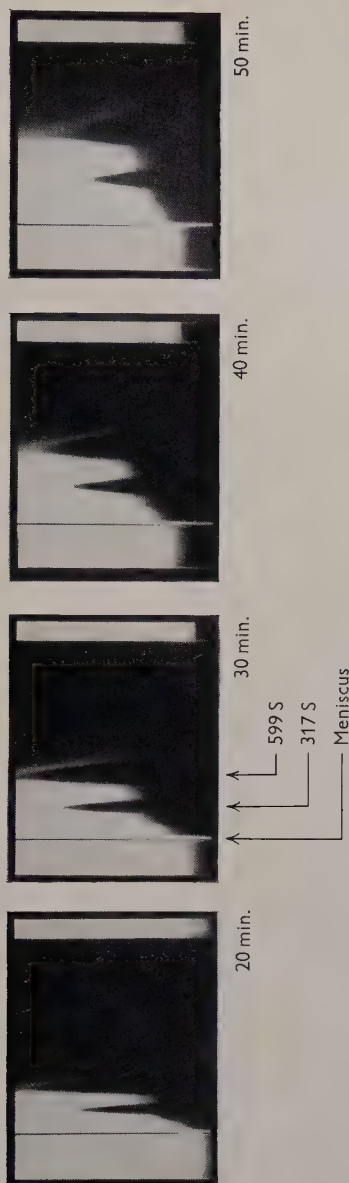
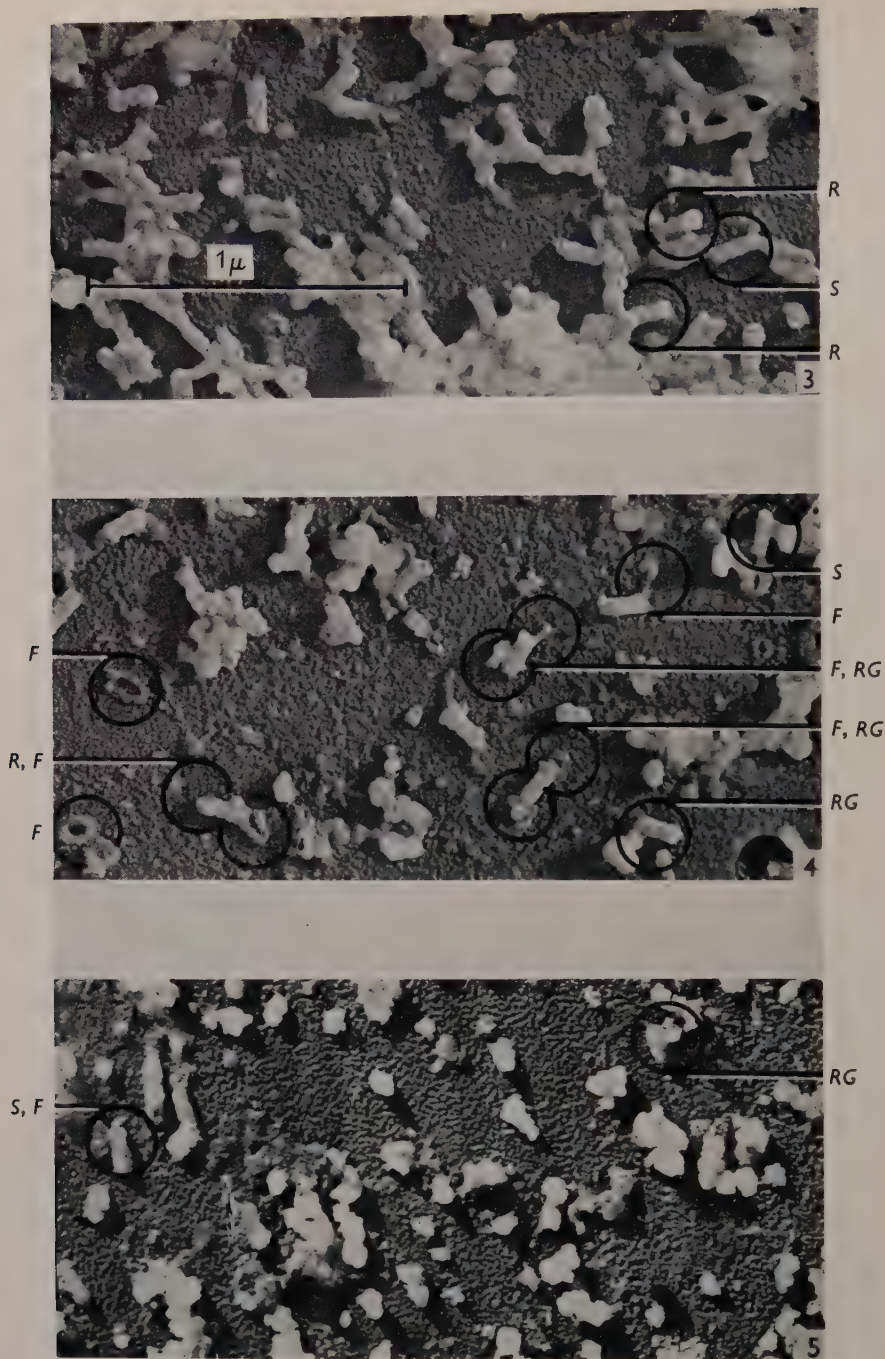


Fig. 2



C. J. BRADISH, J. B. BROOKSBY & J. F. DILLON—VIRUS SYSTEM OF VESICULAR STOMATITIS. PLATE 2

The Production of Hydrogen Sulphide from Thiosulphate by *Escherichia coli*

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SUMMARY: Suspensions of non-proliferating *Escherichia coli* produced H_2S from thiosulphate in the presence of pyruvate or acetaldehyde. Production of H_2S was slight in the presence of α -ketoglutarate and α -ketobutyrate. Organic acids such as malate, fumarate, succinate, lactate, formate and acetate, aldehydes other than acetaldehyde and monohydric alcohols either had no effect or inhibited H_2S production from thiosulphate. H_2S was not produced from sulphite, bisulphite or sulphate, either in the presence or in the absence of the above-named compounds. Crude cell-free extracts of *Escherichia coli* produced H_2S from thiosulphate in the presence of pyruvate. Experiments with dialysed extracts showed that inorganic phosphate, Mg ions and cocarboxylase were essential for H_2S production. Treatment of the extracts with anion exchange resin revealed that in addition, coenzyme A was indispensable for H_2S production from thiosulphate. The addition of DPN to extracts dialysed or treated with anion exchange resin did not influence H_2S production to a marked degree.

Suspensions of non-proliferating cells of various species of Enterobacteriaceae produce H_2S in the presence of such sulphur-containing compounds as cysteine and thiosulphate. The work carried out in this field by many authors has been recently reviewed by Clarke (1953) and by Olitzki (1954). While quantitative differences in the intensity of H_2S production by different micro-organisms are well known, no data exist which explain the mechanism of their H_2S production from inorganic sulphur compounds. The purpose of the present study was to examine the factors governing H_2S production from thiosulphate by non-proliferating organisms and cell-free extracts of *Escherichia coli*.

METHODS

Preparation of suspensions of non-proliferating organisms

Escherichia coli strain B/r was used throughout. The organisms were grown on Difco nutrient agar in Roux bottles. The 18 hr. growth was washed off with distilled water, washed twice in phosphate buffer (pH 6.8) and the suspension brought to the required density as measured in the Coleman Junior spectrophotometer at a wavelength of 550 m μ .

Preparation of cell-free extracts. The organisms were grown as described above. After harvesting and centrifugation they were washed in distilled water and subjected to sonic vibration for 15-20 min. in a Raytheon Magnetostriction Oscillator and then centrifuged at 10,000 r.p.m. for 10 min. The cell-free supernatant solution was used in all experiments. Protein was determined by the

biuret method of Mehl (1945), using a Coleman Junior spectrophotometer at 540 m μ .

Treatment with anion exchange resin. The anion exchange resin 'Amberlite' Ira 410 was charged with 4 % (w/v) sodium chloride until the pH of the effluent was neutral. The resin was then washed with distilled water until no chloride could be detected in the effluent. The resin was added directly to the extracts in equal volume, the suspension stirred for 15 min., centrifuged and the supernatant finally filtered to remove the last traces of resin. This treatment removed coenzyme A as judged by arsenolysis (Stadtman, Novelli & Lipmann, 1951). The cocarboxylase was removed by the same procedure.

Experimental procedure. Sodium thiosulphate was brought in contact with a cell suspension or cell-free extracts in the presence of sodium phosphate buffer (pH 6.8) and incubated at 37° in small test tubes closed with a rubber stopper to prevent any losses of H₂S. After the required incubation, the reaction was stopped by the addition of two drops of 20 % sodium hydroxide and sulphide determined by the method of Delwiche (1951).

RESULTS

Optimal conditions for H₂S production from thiosulphate. Suspensions of non-proliferating organisms were incubated with different amounts of sodium thiosulphate and the quantities of H₂S produced were determined after 2 and 24 hr. of incubation. Under the specified conditions the optimum amount of thiosulphate was about 800 μ mole (Table 1). The optimum pH was of the order of 6.8 (Fig. 1).

Enhancement of H₂S production by glucose. Since Braun & Silberstein (1942) reported that fermentable carbohydrates enhance the production of H₂S from inorganic sulphur compounds by growing organisms, this effect was tested by using non-proliferating cells. Glucose markedly enhanced H₂S production from thiosulphate (Fig. 2), but of several fermentable carbohydrates tested only glucose increased H₂S production, and this to a marked degree (Table 2).

The influence of inhibitors of glycolysis on H₂S production from thiosulphate. To ascertain whether glucose itself or some intermediary product arising from glucose by glycolysis was responsible for the enhancing effect in H₂S production, various known inhibitors of glycolysis were tested. These experiments showed that the various inhibitors reduced or completely arrested H₂S production (Table 3). The almost complete inhibition of H₂S production from thiosulphate by bisulphite, both in the presence and absence of glucose, suggested that the enhancing effect of glucose on H₂S production was due to pyruvic acid or some lower intermediary compound produced in the course of glycolysis, since bisulphite is known to give addition compounds with aldehydes and some ketones. The production of H₂S from thiosulphate by non-proliferating cells in the absence of glucose may thus be explained by the presence of endogenic substrate. Further evidence that without pyruvate there was no H₂S production was obtained in studies with cell-free extracts. Experiments carried out with pyruvic acid showed that this compound strongly stimulated

Table 1. H_2S production from thiosulphate by non-proliferating *Escherichia coli*

Bacterial suspension (organisms/ml.)	Thiosulphate ($\mu\text{mole.}$)	Incubation (hr.)	Total H_2S produced (μmole)
2.5×10^8	800	2	0
2.5×10^8	800	24	0
2.5×10^8	500	2	0
2.5×10^8	500	24	0
2.5×10^8	200	2	0
2.5×10^8	200	24	0
2.5×10^8	100	2	0
2.5×10^8	100	24	0
2.5×10^9	800	2	0.20
2.5×10^9	800	24	0.60
2.5×10^9	500	2	0.15
2.5×10^9	500	24	0.45
2.5×10^9	200	2	0.10
2.5×10^9	200	24	0.40
2.5×10^9	100	2	0.10
2.5×10^9	100	24	0.40
1.25×10^{10}	800	2	0.30
1.25×10^{10}	800	24	2.00
1.25×10^{10}	500	2	0.20
1.25×10^{10}	500	24	1.20
1.25×10^{10}	200	2	0.11
1.25×10^{10}	200	24	1.00
1.25×10^{10}	100	2	0.11
1.25×10^{10}	100	24	0.70

Each tube contained: sodium phosphate buffer (pH 6.8), $100 \mu\text{mole}$; cells and thiosulphate as indicated; total vol., 4 ml.

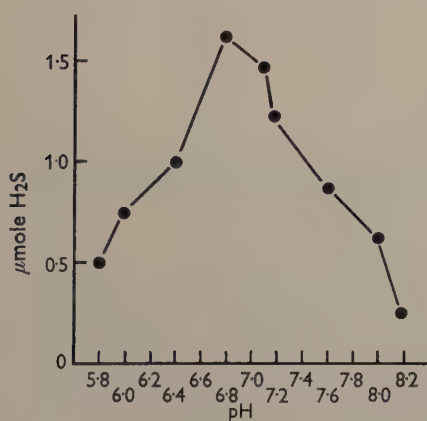


Fig. 1

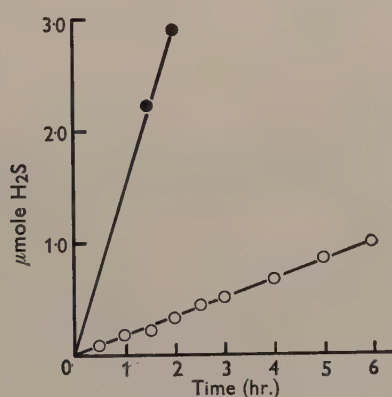


Fig. 2

Fig. 1. Effect of pH value on H_2S production from thiosulphate by non-proliferating *Escherichia coli*. Organism concentration $1.25 \times 10^{10}/\text{ml.}$; thiosulphate, $800 \mu\text{mole}$; sodium phosphate buffer of various pH values, $100 \mu\text{mole}$; final vol., 4 ml.; time of incubation, 4 hr.

Fig. 2. The effect of glucose on H_2S production from thiosulphate by non-proliferating *Escherichia coli*. The reaction mixture contained: glucose, when added, $100 \mu\text{mole}$; thiosulphate, $800 \mu\text{mole}$; sodium phosphate buffer (pH 6.8), $100 \mu\text{mole}$; organism concentration 1.25×10^{10} ; final vol., 4 ml. $-\bigcirc-\bigcirc-$, without glucose; $-\bullet-\bullet-$, with glucose.

H₂S production and the magnitude of this effect was equal to that obtained with equimolar glucose. As expected, the production of H₂S in the presence of pyruvate was not affected by fluoride, in contrast to the results obtained with glucose.

Table 2. *Effect of various fermentable carbohydrates on H₂S production by non-proliferating Escherichia coli*

Carbohydrates (100 μ mole)	H ₂ S produced (μ mole)
Glucose	2.70
Maltose	0.27
Lactose	0.30
Sucrose	0.33
Galactose	0.33
Xylose	0.27
None	0.33

Each vessel contained: carbohydrates as indicated; thiosulphate, 800 μ mole; sodium phosphate buffer (pH 6.8), 100 μ mole; organism concentration, 1.25×10^{10} /ml.; final vol., 4 ml. H₂S determined after 2 hr. at 37°.

Table 3. *The effect of glycolysis inhibitors on H₂S production from thiosulphate in the presence and absence of glucose*

Inhibitors	Concentrations (M)	Glucose present		Glucose absent	
		H ₂ S (μ mole)	Inhibition (%)	H ₂ S (μ mole)	Inhibition (%)
Na bisulphite	0.01	0.10	96	0	100
Na bisulphite	0.001	0.36	89	0	100
Na fluoride	0.01	0.33	90	0.40	—
Na fluoride	0.001	0.73	78	0.40	—
Na iodoacetate	0.01	0.20	94	0.20	50
Na iodoacetate	0.001	0.30	91	0.20	50
None		3.20	—	0.40	—

Each reaction mixture contained: thiosulphate, 800 μ mole; Na phosphate buffer (pH 6.8), 100 μ mole; glucose, when present, 100 μ mole; inhibitors as indicated. Concentration of organisms, 25×10^{10} /ml.; total vol. 44 ml. Sulphide determined after 3 hr.

The effect of various organic acids and alcohols on H₂S production from thiosulphate. Results of the above experiments suggested that H₂S production from thiosulphate is conditioned by the presence of hydrogen donors and thus the effect of adding various hydrogen donors was examined. The results of these experiments (Table 4) showed, surprisingly, that none of the compounds tested enhanced the production of H₂S from thiosulphate. The following organic acids and alcohols were without effect, whether in the presence or absence of glucose: succinate, acetate, formate, lactate, citrate, methanol and propanol. Butanol had a marked inhibitory effect. Malate and fumarate inhibited the reaction completely in the absence of glucose. This result may be tentatively explained by the prevention of endogenic pyruvate formation due to the inhibition of oxaloacetate decarboxylation to pyruvate by malate (Ochoa & Weisz-Tabori, 1948). The same explanation is valid in the case of fumarate

since these acids are readily interconvertible. Another interesting observation that arose from the above experiments was that ethanol in the presence of glucose almost doubled H_2S production. Further experiments showed that only ethanol (among other alcohols examined) enhanced H_2S production from thiosulphate, and then only in the presence of glucose or pyruvate. Ethanol alone or in the presence of all the above-mentioned organic acids had no effect.

Table 4. *The effect of various organic acids and alcohols on H_2S production from thiosulphate in the presence and absence of glucose*

Organic acids and alcohols	H_2S (μmole)	
	Glucose present	Glucose absent
None	3.00	0.20
Pyruvate	3.70	3.20
Fumarate	3.00	0.00
Malate	2.90	0.00
Acetate	2.90	0.20
Formate	3.00	0.19
Lactate	3.00	0.20
Succinate	2.90	0.20
Citrate	3.00	0.20
α -Ketoglutarate	3.70	0.43
α -Ketobutyrate	3.30	0.53
Methanol	3.00	0.20
Ethanol	6.00	0.30
Propanol	2.30	0.20
Butanol	2.00	0.10

Experimental conditions as given in Table 3. The organic acids were neutralized with sodium hydroxide to pH 6.8 prior to addition. Organic acids and alcohols, when added, 100 μmole . H_2S determined after 3 hr.

If an intermediary compound responsible for H_2S production arises it might give rise to ethanol, and addition of ethanol would then cause accumulation of the intermediary compound and thus enhance H_2S production. Although the fermentation processes in *Escherichia coli* leading from pyruvate to acetaldehyde are not known, these experiments suggest that acetaldehyde may be the intermediary compound responsible for H_2S production from thiosulphate, since acetaldehyde enhanced H_2S production to the same degree as glucose or pyruvate (Table 5). Among the inhibitors of glycolysis only bisulphite inhibited H_2S production. No other aldehydes had any stimulatory effect on H_2S production; on the contrary, higher aldehydes from 4-C exerted an inhibitory effect.

H_2S production from various inorganic compounds. Experiments carried out with bisulphite, sulphite and sulphate in the presence and absence of various carbohydrates, organic acids, alcohols and aldehydes, showed no production of H_2S .

H_2S production from thiosulphate by crude cell-free extracts. Production of H_2S from thiosulphate by crude extracts took place only in the presence of pyruvate. In the presence of glucose little if any H_2S was produced. There was

no H_2S production from thiosulphate in the presence of various organic acids and alcohols.

H_2S production by dialysed extracts. Cell-free extracts after dialysis against distilled water for 18 hr. in a cold room at 5° failed to produce H_2S from thiosulphate in the presence of pyruvate unless Mg ions, cocarboxylase and inorganic phosphate were added. The addition of DPN with the above-mentioned cofactors had no appreciable effect on H_2S production (Table 6).

Table 5. (a) *The effect of various aldehydes on H_2S production from thiosulphate.*
(b) *The effect of inhibitors of glycolysis on H_2S production from thiosulphate in the presence of acetaldehyde*

Aldehydes	Inhibitors of glycolysis	H_2S (μmole)
(a) Formaldehyde	—	0.20
Acetaldehyde	—	2.00
Propylaldehyde	—	0.10
Butylaldehyde	—	0.00
(b) Acetaldehyde	Na fluoride 0.01 M	2.30
Acetaldehyde	Na fluoride 0.001 M	2.30
Acetaldehyde	Na bisulphite 0.01 M	0.00
Acetaldehyde	Na bisulphite 0.001 M	0.00

Each test tube contained: thiosulphate, $800\mu\text{mole}$; aldehydes, $80\mu\text{mole}$; sodium phosphate buffer (pH 6.8), $100\mu\text{mole}$; organism concentration $1.25 \times 10^{10}/\text{ml.}$; inhibitors as indicated; final vol., 4 ml. H_2S determined after 2 hr. at 37° .

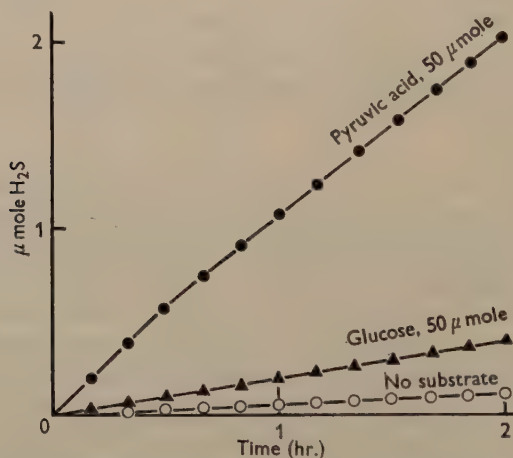


Fig. 3. H_2S production from thiosulphate by crude extracts of *Escherichia coli*. Each test tube contained: extract containing 20 mg. protein; thiosulphate, $300\mu\text{mole}$; sodium phosphate buffer (pH 6.8), $100\mu\text{mole}$; total vol., 3 ml.

H_2S production by extracts treated with anion exchange resin. These extracts, after treatment with anion exchange resin, did not produce H_2S from thiosulphate in the presence of pyruvate unless Mg ions, cocarboxylase, inorganic phosphate and coenzyme A were added. The addition of DPN with the above-mentioned activators did not affect H_2S production appreciably. A typical

experiment is shown in Table 7. In the presence of α -ketoglutarate the results were similar to those obtained with pyruvate, but the amount of H_2S produced was considerably smaller.

Table 6. H_2S production from thiosulphate by dialysed extracts

Cofactors	Inorganic phosphate	H_2S (μmole)
Control before dialysis	Added	2.10
None	None	0.00
None	Added	0.10
MgCl_2 , cocarboxylase	Added	1.80
MgCl_2 , cocarboxylase, DPN	Added	1.60

Each vessel contained: extracts containing 20 mg. protein; thiosulphate, 300 μmole ; pyruvate, 50 μmole ; cocarboxylase, when added, 200 $\mu\text{g.}$; MgCl_2 , if added, 32 μmole ; veronal buffer (pH 6.8), 100 μmole ; sodium phosphate (pH 6.8), if added, 100 μmole ; DPN, when added, 200 $\mu\text{g.}$; total vol., 3 ml. H_2S determined after 2 hr. at 37°.

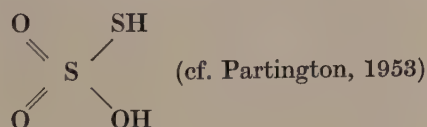
Table 7. H_2S production from thiosulphate in the presence of pyruvate by extracts treated with anion exchange resin

Cofactors	Phosphate	H_2S produced (μmole)
None	Added	0.23
MgCl_2 , cocarboxylase	None	0.29
MgCl_2 , cocarboxylase, coenzyme A, DPN	None	0.31
MgCl_2 , cocarboxylase, coenzyme A, DPN	Added	1.61
MgCl_2 , coenzyme A, cocarboxylase	Added	1.91
MgCl_2 , coenzyme A	Added	0.69
Untreated control	Added	2.30

Experimental conditions as given in Table 6. Coenzyme A, when added, 30 units.

DISCUSSION

The exact mechanism involved in H_2S production from thiosulphate still remains to be elucidated. The requirement for pyruvate (pyruvate could be replaced to some extent by α -ketoglutarate and α -ketobutyrate, and in non-proliferating cell suspensions by acetaldehyde) as well as for some of the cofactors of the pyruvic dehydrogenase system seems to indicate that H_2S formation from thiosulphate is linked to a reductive step, in which pyruvate serves as an obligate hydrogen donor. The specificity of the hydrogen donor appears remarkable. It will be recalled in this connexion that the oxidation of pyruvate in bacteria requires thioctic acid as a primary hydrogen acceptor (reviewed by Gunsalus, 1953). If the formula of thiosulphate is written as follows:



the presence of an S—S bond becomes apparent. It is tempting to speculate that the S—S bond confers on the thiosulphate the capacity to function as a primary hydrogen acceptor, alternative to thioctic acid. This interpretation would readily account for the specificity of hydrogen donor, the failure of other inorganic sulphur compounds to produce H_2S , and the finding that the system mediating H_2S production from thiosulphate, in contrast to pyruvic dehydrogenase, does not depend on DPN as an essential cofactor. It will be pointed out that, according to the scheme of Gunsalus (1954), DPN serves to regenerate the S—S linkage of the reduced thioctic acid. In the reaction described by me the acceptor (thiosulphate) is supplied in excess and therefore the regeneration mechanism is obviously not required.

I wish to express my gratitude to Prof. A. L. Olitzki for having suggested to me the subject of the study, and for constant guidance and encouragement throughout this work, which forms part of a study submitted for the degree of Ph.D. to the Senate of the Hebrew University.

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Inhibition of Coli Bacteriophage T₂ by Apple Pectin

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SUMMARY: Apple pectin in a complex organic medium partly protected *Escherichia coli* strain B from lysis by T₂ phage. It was not bactericidal or virucidal. The rate of adsorption of the phage was unaltered, but part of the initially adsorbed phage could be eluted with distilled water at 0°, as the second irreversible step of adsorption was inhibited by pectin. It was shown in one-step growth and single cell burst experiments that phage multiplication was reduced. The release of any formed phage from the host was not affected. The protective effect of the pectin resulted from the failure of some of the phage particles to penetrate into the host cell and from its action in decreasing phage synthesis in those cells where penetration did take place. It is suggested that this non-specific polysaccharide may exert its protective action because of its polymeric electrolyte nature.

D'Herelle (1926) early on cited the inhibition of phage lysis by gelatin, tragacanth, etc. Bacterial and non-bacterial polysaccharides have been reported to inhibit bacteriophage. Levine & Frisch (1933) demonstrated that extracts from certain salmonella and shigella cultures inactivated phage. Burnet (1934) and Gough & Burnet (1934) confirmed this and found that the phage-inactivating agent was a complex polysaccharide, probably identical with the bacterial surface component on which the phage was adsorbed and which determines the specificity of the phage-host relationship.

Ashenburg, Sandholzer, Sherp & Berry (1940) reported that non-specific polysaccharides like starch, glycogen and gum arabic had an effect similar to specific polysaccharides. They suggested later (Ashenburg, Sandholzer, Sherp & Berry, 1950) that this non-specific inhibition by polysaccharides was not necessarily in conflict with the hypothesis that susceptibility to phage is related to the antigenic structure of the organism. Non-specific polysaccharides may, by virtue of similar chemical structures, compete with the receptor units on the bacterial surface for chemical groupings on the phage and thus partially block lytic activity, a view which was first put forward in relation to bacterial polysaccharides by Gough & Burnet (1934).

The work cited thus far does not describe inhibition of phage multiplication but an inactivation of free phage. Maurer & Woolley (1948), working with *Escherichia coli* and T₂ phage, reported that citrus and apple pectin were non-bactericidal and non-virucidal and did not prevent the adsorption of phage, but prevented the lysis of the cells, perhaps by forming a protective layer around them. Multiplication of phage took place and they compared the effect with a lysogenic system (liberation of phage without lysis as some workers interpreted the phenomenon of lysogenicity at that time).

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Kleczkowski & Kleczkowski (1952) reported that a specific polysaccharide derived from the host bacteria (rhizobium) interfered with the multiplication of a homologous phage. He showed that in the first 3 hr. of incubation in the presence of 0.25 % (w/v) polysaccharides phage synthesis was only about half that in the control. This inhibitory effect disappeared after prolonged incubation.

This paper is concerned with the inhibition of the T_2 coli phage by a non-specific polysaccharide, apple pectin, in an organic medium.

METHODS

Escherichia coli B and phage T_2 were employed, using the routine methods in phage research as described by Adams (1950).

The medium consisted of 1 % peptone, 1 % Lemco meat extract, 0.3 % yeast extract (all Oxoid), 0.5 % sodium chloride in distilled water, pH 7.2. The pectin was dissolved as described by Maurer & Woolley (1948), except that it was found of advantage not to adjust the pH before sterilization. 4 % apple pectin (B.D.H. grade 240) was dissolved in water at 95° by adding it slowly with constant stirring. The resultant solution was then sterilized and the pH adjusted with 2M-caustic soda while still hot. This solution, half diluted with double-strength broth, constituted the pectin broth medium.

The media are referred to in the text as YG broth for yeast-glucose broth, and P broth for YG broth containing the supplement of 2 % (w/v) pectin.

The number of organisms was determined either by direct plating in nutrient agar, or with the aid of a nephelometer (Evans Electroselenium Ltd.).

Intracellular phage. The intracellular phage was determined in the manner described for the T_5 phage by Kay (1952). The organisms were artificially lysed by suspension in a saturated solution of glycine containing 0.015 M-potassium cyanide at 37° for 2 hr. It was found that T_2 phage was rather more affected by glycine and potassium cyanide than T_5 phage, and the incubation time was reduced to 2 hr. instead of 3 hr. as used for T_5 phage.

Elution experiments. To determine whether phage was adsorbed reversibly or irreversibly elution experiments were carried out according to Garen & Puck (1951). T_2 phage was mixed with an excess of organisms at 37° and centrifuged after an adsorption period of 5 min. The separated organisms were then resuspended in double-distilled water at 0°. After 5 min. the organisms were again separated by centrifugation and then the eluted phage determined in the supernatant.

This process was repeated once, and the total of the eluted phage was then expressed as a percentage of the adsorbed phage.

RESULTS

Preliminary experiments confirmed the report of Maurer & Woolley (1948) that apple pectin did not prevent the adsorption of T_2 phage and was non-toxic to *Escherichia coli* B. It was also confirmed that the presence of pectin totally or partially inhibited clearing of cultures of *E. coli* B. by phage T_2 ; but the inhibition was less complete when the cultures were continuously aerated.

Exposure of T_2 phage to apple pectin did not inactivate it, for it gave the same plaque count after being suspended in P broth at 37° as before this treatment.

Rate of adsorption. While it was confirmed that adsorption did take place in the presence of pectin no data were available regarding the rate of adsorption. If adsorption were substantially retarded it could account for at least some of the protective action of pectin.

8×10^7 cells/ml. were infected with 8×10^7 phage/ml. in YG broth and P broth at 37° and kept for 5 min. after mixing (the rate of infection was the same as used for the single cell burst experiments). The unadsorbed free phage was then determined in the usual way.

The velocity constant K was practically identical for both media, namely, 3.9×10^{-9} ml./min. for YG broth, and 4.2×10^{-9} ml./min. for P broth.

Experiments with high concentrations of organisms (10^9 /ml.) and a low rate of phage infection (10^5 /ml.) as used in the elution experiments, confirmed the identical rate of adsorption in the two media.

One-step growth experiments and intracellular phage. Since the latent period of the T_2 coli phage at 37° was too short to permit the taking of sufficient samples for the determination of intracellular phage, the incubation temperature was lowered from the optimum of 37 to 30° .

A culture of *Escherichia coli* B, grown in YG broth to a concentration of 10^7 organisms/ml., was centrifuged and washed twice with distilled water. The packed cells were resuspended in YG broth and P broth respectively and incubated at 37° for 15 min. and then infected with 10^7 phage/ml. After an adsorption period of 5 min. the free, unadsorbed, phage was eliminated by centrifuging a 1:100 dilution of the infected culture and decanting the clear supernatant. The sediments containing the infected bacteria were resuspended in fresh media. The appropriate dilutions were then assayed for total and intracellular phage.

The glycine KCN method for artificial lysis (Kay, 1952), which was originally used for the T_5 phage, proved to be applicable for the estimation of intracellular T_2 phage as shown in Fig. 1. Very little intracellular phage was detected up to 15 min. after infection in YG broth which indicates, according to current conception of phage multiplication, that the adsorbed phage had been broken down inside the cell. Apparent burst sizes (i.e. the ratio of phage count at the end of the rise period to the initial phage count) from 120 to 150 phage particles were regularly obtained in this medium.

Fig. 2 shows a one-step growth curve in the presence of pectin. Such experiments gave apparent burst sizes of 10–30 phage particles. The level of the demonstrable intracellular phage during the early latent period was very much higher than in the absence of pectin. Much of the adsorbed phage was not broken down and presumably did not penetrate into the cell. The first noticeable increase in both total and intracellular phage was delayed, giving a longer latent period. The phage count at the end of the burst period was never increased by dissolution of the cells in glycine. The low phage yields obtained were, therefore, not due to any action of pectin which prevented the cells from liberating phage.

While the low phage yield in the presence of pectin was at least partly due to the failure of the phage to penetrate the host cell, it was found in one-step growth and in single cell burst experiments that the phage yield was also reduced when the adsorption took place in YG broth with subsequent dilution and growth in P broth.

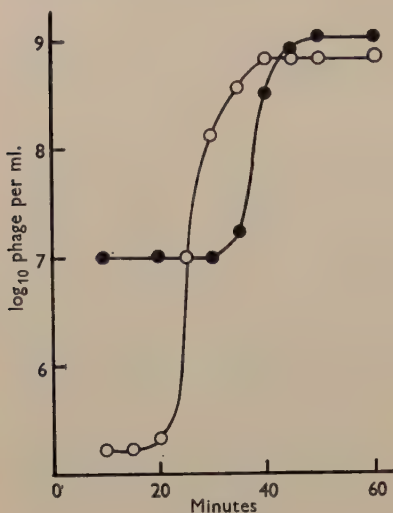


Fig. 1

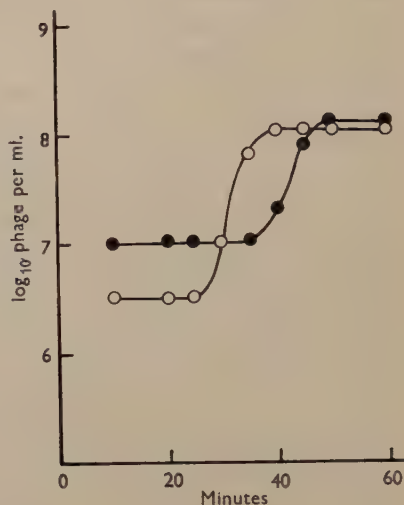


Fig. 2

Fig. 1. Phage multiplication in YG broth; one-step growth curve. Total phage/ml., ●; intracellular phage/ml., ○.

Fig. 2. Phage multiplication in P broth, one-step growth curve. Total phage/ml., ●; intracellular phage/ml., ○.

The data show that the yield of phage was appreciably reduced in the presence of pectin but they do not reveal how many of the infected cells go on to burst and what the range is in individual burst sizes.

Single cell burst experiments. The input ratio of phage to bacterium was 10:1 with an adsorption period of 5 min. at 37°. The free, unadsorbed, phage was again eliminated by sedimenting the infected cells and decanting the supernatant. The percentage of adsorption was 80 % under these conditions.

In YG (Table 1) the average burst size and broad range of distribution of burst sizes were as expected. In the presence of pectin throughout the whole period of adsorption and growth the individual burst sizes were not only much smaller but a good proportion of the positive plates always contained single plaques. It was, of course, ascertained that the single plaques did not originate from any free phage particles which may have been distributed in the individual 0.5 ml. samples with the infected cells during the patent period. The average burst size in this particular experiment was either 17 or 47/cell according to whether the positive plates showing single plaques are included or excluded in the calculation.

As the phage yield was influenced by the adsorption process in the presence of pectin it was necessary to determine the average burst size after adsorption

in YG broth and transfer of the infected cells to P broth. The average burst size was lower than in YG broth and practically identical with that found in the experiments in P broth if one disregards the single plaque plates in the calculation of the latter. It is evident that the single plaque plates did not occur unless pectin was present during the adsorption period.

It can be inferred from these results that the single plaques originated from infected cells which had failed to produce a burst.

Table 1. *Single cell burst experiments*


Adsorption medium	Growth medium	Individual burst sizes	Average burst size
YG broth	YG broth	1, 80, 81, 88, 104, 107, 122, 123, 161, 168, 177, 215, 223, 284, 340, 343, 400	150
P broth	P broth	1, 1, 1, 1, 1, 1, 1, 1, 7, 14, 28, 94, 95	17 (47)*
YG broth	P broth	10, 10, 45, 45, 50, 62, 64, 65, 66, 101, 132	54

* Disregarding the single plaques.

Elution experiments

Both the determination of intracellular phage during the early latent period in the one-step growth experiments and the occurrence of positive plates with single plaques in the single cell burst experiments suggested that the pectin prevents the penetration of the phage into the host. Garen & Puck (1951) have shown that distilled water at 0° will elute phage which has been adsorbed under certain environmental conditions, as at a low temperature and in medium of suboptimal salt concentration. If the T₂ phage does not penetrate the host in P broth it is to be expected that it can be eluted.

Table 2. *Elution experiments*

Adsorption medium	Percentage of phage adsorbed	Percentage of adsorbed phage eluted
YG broth* 	96	3
P broth	96	62
P broth + 0.1 M-NaCl	94	58
P broth + 0.5 M-NaCl	94	5

* Contains 0.1 M-NaCl.

10⁹ cells/ml. in YG broth and P broth were infected with 10⁵ phage/ml. at 37°, kept for 5 min., centrifuged and eluted with distilled water according to Garen & Puck. YG broth itself contains 0.1 M-sodium chloride and is, therefore, an optimum adsorption medium for T₂ phage with the given excess of cells (over 90 % adsorption). As expected almost no phage was eluted after adsorption in this medium (Table 2) because the adsorption was irreversible.

In the presence of pectin, however, a large proportion of phage was eluted, proving that a great part of the phage was reversibly bound to the host.

Decreasing concentrations of ions in the adsorption medium (e.g. below 0.1 M-NaCl) make the adsorption process increasingly more reversible. It is, therefore, possible that pectin acts as a chelating agent, reducing the ion concentration and permitting the elution of the phage. The addition of sodium chloride to the pectin medium might, therefore, be expected to prevent elution, and Table 2 shows that it has this effect. But no inference on the role of pectin in such a modified medium can be made, since pectin itself becomes altered and unstable after the addition of more than 0.1 M-NaCl. It is, therefore, not clear from these experiments whether NaCl acts by supplying the need of ions for the irreversible adsorption process or whether it destroys the inhibiting action of pectin itself.

DISCUSSION

Various explanations have been put forward to interpret the effect of non-specific polysaccharides on bacteriophage action, but so far as pectin is concerned, little experimental evidence is available. The results presented show that apple pectin does not itself inactivate phage, does not prevent the adsorption of phage to its host or reduce the rate of phage adsorption, and that it does not interfere with any liberation of synthesized phage from the cell.

Its action can be explained in the light of the present results in two ways. First, inhibition of the second step of adsorption, which is known to kill the host. This failure to complete the adsorption process in the presence of pectin was demonstrated by the elution of the phage, its recovery by artificial lysis of the infected cells during the early latent period and the appearance of single plaques in the single burst experiments. All these experiments showed that a large part of the phage remained extracellular in the presence of pectin. Secondly, the low rate of multiplication in P broth, even when adsorption took place in Y-D broth in the absence of pectin. This showed that the phage synthesis itself must be affected by the pectin.

The elution of phage adsorbed in the presence of pectin and its prevention by high concentrations of NaCl in the adsorption medium could be explained by the sequestering effect of pectin which could act as a polymeric electrolyte. Such action of the pectin would, however, only be possible at a limited range of salt concentration, for it is known that neutral salts greatly influence the electrolytic nature of charged polymers themselves.

Another possible explanation may be considered, namely, that the pectin as a polyelectrolyte might be absorbed directly to the bacterial surface, interfering with both the adsorption and the multiplication of the phage. Bichovsky-Slomnitzky (1953) demonstrated that the positively charged polyelectrolyte polylysine attached itself to *Escherichia coli*. It may well be that the same could happen with the negatively charged polyelectrolyte given suitable ionic concentration in the medium.

Pectin, of course, is not a definite chemical entity and pectins from different sources vary considerably in their chemical composition. They have been found to vary also in their inhibition of coli T₂ phage (e.g. elutions of up to 95% have been found with one batch of apple pectin).

It is to be expected, of course, that the chemical constitution of the pectin will influence its action in phage inhibition, and further explanations of this inhibition are likely to be found in the nature and extent of the active radicle groups present in the pectin.

I wish to thank the Directors of Benger's Ltd. for their permission to publish this paper, and to express appreciation for the kind encouragement given by the local Director, Mr C. F. Stenson.

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Identification of *Azotobacter* Species by Fluorescence and Cell Analysis

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SUMMARY: Eight strains of *Azotobacter agile* and *A. vinelandii* were studied for their ability to elaborate water soluble compounds with a fluorescence that would characterize each when observed under ultraviolet light of 3600 Å. It was found that the material produced by *A. agile* fluoresced white, whereas that produced by *A. vinelandii* fluoresced green. Additional studies with iron and molybdenum showed that molybdenum enhanced synthesis of fluorescent material in both species and iron appeared to quench the fluorescence. A pH/fluorescence curve for the fluorescent material of each species showed that, although similarities were evident, sufficient difference existed to permit recognition of each. Analyses of dried cell material revealed a much higher protein content in *A. agile* than in *A. vinelandii*, but the amount of one of their amino acids, lysine, was essentially the same, on the basis of protein, for each species.

Production of fluorescent pigments by the azotobacters has long been known. Even Beijerinck's (1901) first description of *Azotobacter agile* mentioned the existence of a greenish yellow pigment resembling that in fluorescent bacteria. Since that time, references to pigments of this nature have been repeatedly mentioned in connexion with azotobacter, and occasionally they have been of taxonomic significance. In none of these reports was there ever mentioned the use of ultraviolet light to observe the characteristic fluorescence. It is assumed, therefore, that in most, if not all, instances the observations for fluorescent pigments were made in normal daylight. Johnstone (1955) reported that the use of ultraviolet light of 3600 Å was useful in observing the production of fluorescent substances produced by *A. agile* Beijerinck and *A. vinelandii* Lipman, and this prompted us to investigate other strains of each species to see if they too would emit fluorescent light with a characteristic colour when excited by ultraviolet light. The present paper deals with this investigation, and also presents an analysis of cell material, contributing further information which we hope will be useful in separating *A. agile* from *A. vinelandii*. A recent paper (Schutter & Wilson, 1955) also pointed out some new biochemical approaches toward taxonomic identification of these two species.

METHODS

Ultraviolet light observation. Sixteen strains obtained from various laboratories, and including our own isolates, are listed in Table 1. Burk's nitrogen-free agar (Wilson & Knight, 1952) with 2% (w/v) glucose was used for growth, and after 4 days of incubation at 30° the cultures were observed in the dark under ultraviolet light of 3600 Å for colour differences in fluorescence.

Influence of iron and molybdenum. An experiment designed to show the effect of iron and molybdenum on the production of the characteristic yellowish green pigment by *Azotobacter vinelandii* (Wilson & Knight, 1952) involved liquid cultures with increasing amounts of iron in the form of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ or molybdenum in the form of MoO_3 , or both. The iron was incorporated in Burk's nitrogen-free medium in 0, 0.01, 0.1, 1.0 and 10 p.p.m. The molybdenum was

Table 1. *Source of strains of azotobacter*

<i>Azotobacter agile</i>				<i>Azotobacter vinelandii</i>			
Strain	Laboratory	Location	No.*	Strain	Laboratory	Location	No.
103	J. Smit	Holland	†	102	ATTC	Washington	9046
127	J. Smit	Holland	S 1	107	H. L. Jensen	Denmark	M 2
106	H. L. Jensen	Denmark	S	120	P. W. Wilson	Wisconsin	0
122	P. W. Wilson	Wisconsin	4.4	121	P. W. Wilson	Wisconsin	3
123	P. W. Wilson	Wisconsin	S-2	129	C. B. van Niel	California	MB 3.1
124	P. W. Wilson	Wisconsin	S-4	1	D. Johnstone	Vermont	—
130	C. B. van Niel	California	MB 4.4	3a	D. Johnstone	Vermont	—
131	C. B. van Niel	California	MB 4.5	3b	D. Johnstone	Vermont	—

* Indicates donor's designation.

† Culture referred to by J. Smit (1954), in *J. gen. Microbiol.* 11, vii.

present in 0, 0.001, 0.01, 0.1 and 1.0 p.p.m. Duplicate series of these media were prepared. One series was inoculated with a strain of *A. agile*, the other series with a strain of *A. vinelandii* which were incubated at 30° on a rotary shaking machine for a period of 5 days. It was reasoned that if iron and molybdenum influenced the production by *A. vinelandii* of pigment visible in daylight, it might well influence the fluorescent material.

Influence of pH value on fluorescent pigments. It has been shown to be of interest to obtain a pH/fluorescence curve or the amount, expressed in per cent, of fluorescent light emitted at different pH values, to characterize partially a fluorescent compound (Goodwin, 1953). The organisms were removed from a culture by centrifugation, the supernatant fluid placed in buffer at different pH values, and the relative fluorescence was read on a Klett fluorimeter. The procedure followed was essentially the same as employed by Kavanagh & Goodwin (1948), but with buffer solutions made from Coleman buffer tablets. The instrument was adjusted to read 100 on a linear scale with a quinine standard (1 mg. quinine sulphate/l. of 0.1 N- H_2SO_4). With the sample showing maximum fluorescence adjusted to 100 %, the other values were read relative to it in per cent.

Analysis of dried cell material. In an effort to characterize further the two species, *Azotobacter agile* and *A. vinelandii*, it was thought desirable to see whether or not there were any differences in protein content. A series of analyses was obtained on dry cell material. Two different strains of each species were grown, in duplicate lots, in 6 l. fermentors. Burk's nitrogen-free medium was employed, and the cultures aerated at approximately 6 l./min. After 6 days of incubation at 30° the cell material was harvested by a Sharples centrifuge and air-dried at 70°. The cell material was analysed in duplicate, thus

making a total of sixteen samples for analysis. Total nitrogen was determined by a micro-Kjeldahl procedure and the total protein was computed from this. The ash content was desired to show that organism weight was not greatly affected by salts, and was obtained by ignition at 600° for 4 hr. The amount of lysine was desired to know whether or not the protein of related species is similar. Lysine was selected arbitrarily because it is known to be synthesized in appreciable quantities by organisms of this genus, and because reliable microbiological procedures for its assay are available. The lysine determinations were made microbiologically by assaying hydrolysed cell protein with *Leuconostoc mesenteroides* and measuring the response turbidimetrically with a Coleman Universal colorimeter.

RESULTS

Observations in ultraviolet light

The observation of the cultures in Table 1 under ultraviolet light revealed a striking difference between the two species. The strains of *Azotobacter agile* showed soluble material which fluoresced a brilliant white, whereas the strains of *A. vinelandii* showed soluble material which fluoresced green. Most strains of both species developed a yellowish green pigment which was visible in normal daylight; this was more pronounced with *A. vinelandii*. Further studies with liquid cultures paralleled the observations with those grown on an agar medium. However, the development of a yellowish green pigment in *A. agile* did not alter the colour of brilliant white fluorescence observed under ultraviolet light. In fact, one strain produced a water-soluble dark brown pigment, but the culture still fluoresced like the other strains of *A. agile*. All these strains of *A. agile* failed to grow on mannitol as the sole source of carbon, thus confirming the observation of Kluver & van den Bout (1936). On the other hand, all strains of *A. vinelandii* grew luxuriantly on this substrate, confirming the report of Schutter & Wilson (1955).

Influence of iron and molybdenum

The effects of adding iron or molybdenum were twofold. In *Azotobacter vinelandii*, iron appeared to stimulate cell synthesis to a marked degree, but quenched the production of the yellowish green pigment. In the higher concentrations of iron a brownish pigment developed. Molybdenum, on the other hand, appeared to favour the synthesis of the yellowish green pigment but was not particularly stimulatory to cell synthesis. Obviously, it is unlikely that the media without added iron or molybdenum were entirely free of either substance. When this series of flask cultures was viewed under ultraviolet light, the brilliant green fluorescence was most intense in those flasks containing added molybdenum but not iron. It was still observable in molybdenum-containing cultures which also contained 0.1 p.p.m. iron, but was completely quenched by 1.0 p.p.m. iron at all concentrations of molybdenum. A moderate amount of growth and fluorescent material was formed in the absence of added iron and molybdenum.

Identical results were obtained with *Azotobacter agile* except that little or no pigment was observed in daylight. Under ultraviolet light the brilliant white fluorescence showed the same pattern of response to iron and molybdenum as was found for *A. vinelandii*. Schutter & Wilson (1955), reporting on the cultivation of each species in the absence of added iron or molybdenum, stated that *A. vinelandii* strains turned the medium a light green, whereas the colour formed by *A. agile* was less intense. Had they observed these under ultraviolet light they probably would have seen considerable fluorescence in both species.

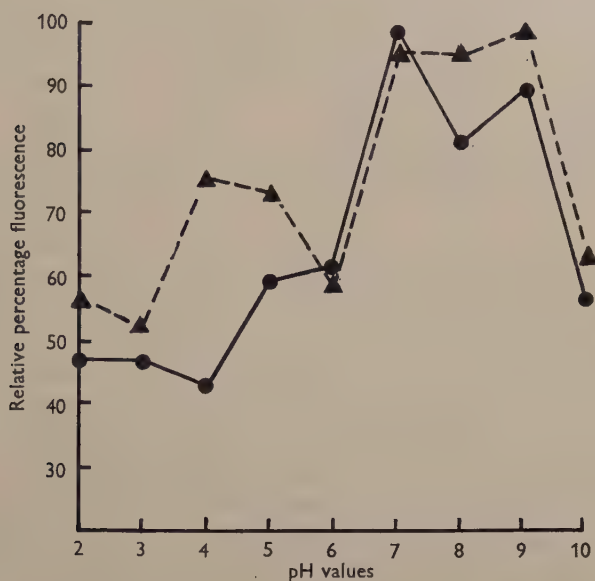


Fig. 1. The pH/fluorescence curves for the culture fluids of cultures of *Azotobacter vinelandii* 3a and *A. agile* 106. ●—●, 3a; ▲—▲, 106.

Influence of pH value on fluorescent pigments

Fig. 1 shows the percentage of light emitted by the culture filtrate of *Azotobacter vinelandii* 3a when adjusted to different pH values and read on a Klett fluorimeter. The peak of light emission appeared at pH 7 and decreased above and below this pH value. However, there was still considerable fluorescence observable by the naked eye when viewed under ultraviolet light at pH values as low as 5 and as high as 10. Though the fluorimeter did not distinguish between the two colours emitted by the two species, it did yield a pH/fluorescence curve for the culture filtrate of *A. agile* (Fig. 1), which showed a remarkable similarity to that of *A. vinelandii*, but sufficiently different to permit a distinction to be made between the two micro-organisms. The culture filtrate of *A. agile* showed a secondary peak of fluorescence at pH 4 not found in the material elaborated by *A. vinelandii*.

Analysis of cell material

Table 2 shows the average results of analyses for protein (based upon micro-Kjeldahl total nitrogen), ash and lysine. The amount of cell protein based upon cell weight was markedly different for the two species, *Azotobacter agile* containing almost twice the amount found in *A. vinelandii*. Since the percentage

Table 2. Average analyses of dried cell material

Organism	Protein (%)	Ash (%)	Lysine*		
			($\mu\text{g./mg. CM}$)	($\mu\text{g./mg. P}$)	(%)
<i>A. agile</i> 103	31.1	7.3	16	51	5.1
<i>A. agile</i> 106	29.4	6.2	13.6	46	4.6
<i>A. vinelandii</i> 102	16.7	7.0	8.3	49	4.9
<i>A. vinelandii</i> 3a	13.8	4.0	7.2	51	5.1

* Designations: CM = cell material, and P = protein.

of ash was not great and the amounts were similar, salts can be ruled out as accounting for a variation in cell weight. The protein is based upon total nitrogen, and as a fixed form of nitrogen was not added to the medium, and as the cell slime does not contain nitrogen, it can be assumed that figures for protein/unit weight of cell material represent the protein of the organisms. The apparent differences in amount for the two species may be due to a difference in the amount of polysaccharide slime in the dried cell material, and hence may represent a difference in slime synthesis. Whatever the reason, the difference is sufficiently pronounced to offer an additional character for taxonomic separation of the two species. Though the amount of lysine/unit weight of cell material differed with each species, as might be expected from the protein determinations when expressed per unit weight of protein, the values are remarkably similar and show that, though protein may be of different quantity per unit weight of cells, at least one of the individual amino acids occurred in the same proportion in both species.

DISCUSSION

The suggestion was made (Johnstone, 1955) that the production of fluorescent substances by micro-organisms might be of considerable taxonomic significance if observations be made with a suitable source of ultraviolet light. This is now amplified by these investigations, which have shown that all *Azotobacter vinelandii* strains so far tested, when grown in a medium low in iron, produce material which fluoresces green under ultraviolet light, while under the same conditions *A. agile* strains fluoresce white. It is of additional importance that none of the 16 strains in Table 1 have lost this ability nor have any other strains of this genus been shown to acquire it during an observation period of one year. The quantity of fluorescent material elaborated by each species varied from strain to strain. These materials produced by each species are not a temporary phenomenon during the growth of the cultures, but once formed are permanent and

stable to heat. They withstand autoclaving without apparent alteration. It might be pointed out that we have received two additional strains labelled *A. agile*, neither of which produces any fluorescent material. Both these strains appear to have all the characteristics of *A. agile*, including an inability to grow on mannitol as the sole source of carbon. It is not surprising to find strains of any group of micro-organisms which differ from the normal pattern by not forming some metabolic product. If they had produced the fluorescent substance characteristic of the other species, *A. vinelandii*, while retaining other characteristics of *A. agile*, there would be some concern. Therefore, it is felt that the use of ultraviolet light, while not always sufficient in itself, is of additional help in separating these two species.

It is not conclusive on the basis of four strains, but perhaps indicative, that *Azotobacter agile* produces considerably more protein on the basis of cell weight than the amount synthesized by *A. vinelandii* under the experimental conditions used. This means that micro-Kjeldahl analysis for total nitrogen of each species will reveal a difference which again may be helpful in separating the two species.

We are indebted to R. H. Goodwin, Connecticut College, New London, Connecticut, for his kindly advice and for permitting us the use of his fluorimeter for the pH/fluorescence curves. We are also indebted to J. Smit of Wageningen, Holland; H. L. Jensen of Lyngby, Denmark; M. Alexander of University of Wisconsin; and C. B. van Niel of Pacific Grove, California, for kindly supplying certain cultures during the course of this work. The study was supported by the State of Vermont; this is a contribution from the Vermont Agricultural Experiment Station, Journal Series Paper no. 56.

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Studies with a *Pseudomonad* able to Grow with Creatine as Main Source of Carbon and Nitrogen

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SUMMARY: An organism able to grow in a simple medium with creatine as the main C and N source was isolated from garden soil and subsequently identified as *Pseudomonas ovalis* Chester. Organisms harvested from a creatine-containing medium destroyed creatine with the uptake of O₂ and the formation of CO₂, NH₃ and urea. With the exception of urea the quantities of reactants fell short of those required by theory, and only part of the deficiency could be attributed to oxidative assimilation. Optimal conditions for the growth of active organisms and for the destruction of creatine were determined. In these conditions the organisms destroyed, in addition to creatine, only arginine and agmatine from a variety of compounds tested; compounds not attacked included creatinine and glycoeyamine. Ability to oxidize creatine was partially lost during repeated washing and storage of the organisms, and was inhibited by *p*-chloromercuribenzoic acid and fatty acids.

Micro-organisms able to use creatine as a substrate for growth have been known for many years (den Dooren de Jong, 1926; Twort & Mellanby, 1912). The classical work of Dubos and Miller revealed further species adaptively capable of destroying creatine (as well as creatinine and other substrates) in washed suspension (Miller & Dubos, 1936; Dubos & Miller, 1937, 1938). The use of a washed suspension of one of these species allowed a discrimination to be made between creatinine and other Jaffe-positive material in human blood. More recently organisms which can similarly use for growth creatine or creatinine as sole source of carbon and nitrogen, and which can as suspensions of the organisms degrade these two compounds, have been isolated from a number of sources (Beard, 1943, 1944; Kopper & Beard, 1947; Roche, Girard, Lacombe & Mourgue, 1948).

In 1949 it became apparent to workers in this Department that the use of an enzyme system specifically capable of destroying creatine would materially aid in investigations on the excretion of this compound. Unfortunately, none of the organisms mentioned above possesses the necessary degree of specificity. All appear to destroy creatinine, and some also to degrade guanidine compounds which give a colour in the method (Ennor & Stocken, 1948) used for creatine estimation. It was decided, therefore, to attempt to isolate a micro-organism, washed suspensions of which could destroy creatine only. In this paper are reported some of the properties of a pseudomonad isolated by specific enrichment; a preliminary account of it has already been given (Nimmo-Smith, 1949).

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METHODS

Organism. The organism (whose isolation is described in Results) was maintained by monthly transfer on tryptic-casein agar slopes incubated for 24 hr. at 30°. It was provisionally identified as *Pseudomonas eisenbergii* (*P. non-liquefaciens*), but a more thorough examination by Dr S. T. Cowan leads him to think that it is a strain of the related species *P. ovalis* Chester. A culture has been deposited as no. 7914 in the National Collection of Type Cultures.

Medium. The basal medium used throughout contained: KH_2PO_4 , 1.9 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 9.3 g.; NaCl, 5 g.; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 100 mg.; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 10 mg.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 200 mg.; $\text{FeSO}_4 \cdot 6\text{H}_2\text{O}$, 10 mg.; yeast extract (Difco), 0.5 g.; distilled water to 1 l.; pH 7.0. To this basal medium was added the desired growth substrate (creatine, arginine, etc.) at 2.5–5.0 g./l.

Growth experiments. The above medium (or indicated modifications of it) was distributed (for 5 ml. final volume) in 50 ml. Erlenmeyer flasks. After autoclaving for 7 min. at 10 lb./sq.in. each flask was inoculated with 0.5 ml. of a 1/100 dilution of a 24 hr. culture in the basal medium+creatine. When a non-adapted inoculum was wanted about the same number of organisms from a tryptic casein agar slope culture were used. All incubations were at 30°. Inability to grow in any particular set of conditions was not concluded until after at least 72 hr. In later experiments, medium (final volume 4.0 ml.) was incubated in sloped $6 \times \frac{3}{4}$ in. test-tubes and growth was assessed with a photoelectric colorimeter (Evans Electroselenium Ltd.; EEL.) with a neutral density filter.

Preparation of suspensions of organisms. The medium (100 ml.) containing 0.5 g. creatine or other substances stated, was distributed in Roux bottles and autoclaved for 7 min. at 10 lb./sq.in. Each bottle was inoculated with 0.1 ml. of a culture of the organism in the same medium and incubated in the horizontal position at 30°. After 20–24 hr. the organisms were harvested on the centrifuge, washed with 0.02M-phosphate buffer pH 7 (50 ml.) and finally suspended in 5 ml. buffer or water to give a concentration of *c.* 5 mg. dry wt. organisms/ml. The relationship between dry weight and instrument reading of either the EEL. or the Hilger Spekker photoelectric colorimeter was established. A reading on the EEL. instrument of 23.5 corresponded to a suspension of 0.5 mg. dry wt. organisms/ml. Suspensions prepared in this way were often used at once, but could be stored at +2° for at least a week in semi-anaerobic conditions with only slight loss of activity.

Activity of suspensions. Two main methods were used to explore the specificity of the harvested organisms and to characterize the process by which creatine was destroyed.

(1) One of the objects of this work was to isolate an organism which could destroy creatine but not other compounds which give a colour with diacetyl and α -naphthol in alkaline medium (Voges & Proskauer, 1898; Barritt, 1936). In all the earlier experiments, therefore, organisms were shaken with a known concentration of each compound to be tested, and the amount removed

determined colorimetrically. Once they had been established, the conditions optimal for creatine removal were used in the specificity tests. Thus a volume of 4.0 ml., containing 0.5–2.0 mg. dry wt. organisms, 1.0 mg. creatine or an equivalent weight of one of the other substrates, and buffered at pH 7.8 with 0.05 M-phosphate, was shaken for 30–120 min. at 30°. The reaction was stopped by adding 6.0 ml. of a solution of *p*-chloromercuribenzoic acid (0.67 g./l.).

The organisms were removed by centrifuging and the amount of substrate which remained in the supernatant was estimated by the method of Eggleton, Elsdon & Gough (1943), as modified by Ennor & Stocken (1948). For each compound tested the rate of colour-production and the relationship between concentration and colour intensity were established. The colour produced by creatine and by dimethylguanidine was measured 20 min. after the addition of reagent; the colour produced by all other compounds was read at 40 min. Creatinine was estimated by the Jaffe reaction.

(2) In the second method the conventional Warburg manometric technique was used, both to confirm some of the findings in the specificity tests and also to study more closely the kinetics and products of creatine catabolism. Usually, 1.0 ml. suspension (equivalent to *c.* 5 mg. dry wt. organisms) and 1.0 ml. 0.2 M-phosphate buffer, pH 7.8, were placed in the main compartment of the flask and 0.5 ml. 0.033 M-creatine tipped in from the side-bulb after equilibration at 30°. When ammonia was to be estimated the reaction mixture was buffered at pH 7.0. The reaction was usually stopped by heating the contents of the flasks for 5 min. in a boiling water-bath; after centrifuging the supernatant fluid was used for analysis.

Creatine was estimated as above. It was found (D. D. Woods, unpublished observations) that ammonia could be estimated in the presence of urea by distilling in the Markham (1942) apparatus after the addition of 0.35 M-borate buffer of pH 8.5. Urea was determined by the difference in ammonia content of the solution before and after incubation with urease.

Chemicals. Some of the compounds listed in Table 5 were gifts generously made. We are indebted to Imperial Chemical Industries (through the courtesy of Dr F. L. Rose) for compounds 2 to 8, to Dr H. King for compounds 9 to 12 and 15 to 17, and to Dr P. C. Spensley for compound 13. We are grateful also to Dr L. A. Stocken for a generous supply of *p*-chloromercuribenzoic acid. The other materials used were of commercial origin and, with two exceptions, were not further purified. Creatine was recrystallized according to Hunter (1928), and the sample of decamethylenediguanidine was converted from the carbonate to the hydrochloride.

RESULTS

Isolation of the organisms

The medium originally used was similar to that described above, but without yeast extract and containing 0.5 % (w/v) creatine; it was buffered to pH 6, 7 or 8. Samples (25 ml.) in 250 ml. Erlenmeyer flasks were inoculated with *c.* 1 g. garden soil from several sources, and incubated at 18, 25, 30 and 37°. After

incubation for 3 days diffuse microbial growth was seen in those flasks which had been inoculated from ground recently used as a chicken-run. Growth was heaviest at 25 and 30°, but seemed to be unaffected by the pH range covered. Morphological appearance and the production of a greenish yellow pigment with an electric-blue fluorescence in ultraviolet light suggested that it was the same organism growing in each flask. The culture obtained at 30° and pH 7 was subcultured five times in these conditions, with 2 days of incubation on each occasion. The fifth subculture was plated on the creatine-containing medium solidified with 1.5 % (w/v) agar, and single colonies isolated. Tested on a variety of media the organism behaved as though in pure culture and was considered to be in a state suitable for further investigation.

As stated above, the organism is probably a strain of *Pseudomonas ovalis* Chester. It was isolated only from soil taken from this one situation; even after 14 days of incubation flasks inoculated with soil from other sources showed no visible growth.

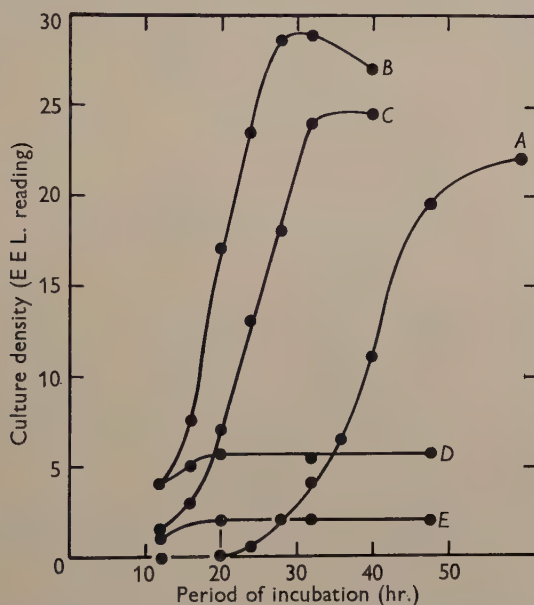


Fig. 1. Effect of yeast extract and of casein hydrolysate on growth of *Pseudomonas ovalis*. Basal medium supplemented with 5 mg./ml. creatine (A), creatine + 0.5 mg./ml. Difco yeast extract (B), creatine + 0.1 mg./ml. acid-hydrolysed casein (C), yeast extract alone (D) or acid-hydrolysed casein alone (E). Incubation at 30° in sloped test-tubes.

Some growth properties of the organism

The organism grew very well on all the common laboratory media tested, but only under aerobic conditions.

Effect of yeast extract. In the simple medium with creatine as only added organic constituent growth was less rapid than in more complex media. Inclusion of 0.05 % (w/v) yeast extract decreased the lag period and somewhat increased the rate and extent of growth (Fig. 1). Yeast extract was normally

included in the medium used for growth tests and always when the cultures were to prepare suspensions of organisms.

An attempt was made to discover what component of the yeast extract was responsible for its stimulatory effect. Several vitamins were tested, singly or in groups, at various concentrations. Neither biotin, pyridoxin, pyridoxal, nicotinic acid, pantothenic acid, riboflavin, thiamine nor *p*-aminobenzoic acid improved growth. Acid-hydrolysed casein caused a similar marked decrease in the lag period (Fig. 1) which could not be traced to the effect of any particular amino acid. The single addition of almost any amino acid has some effect; glutamic acid (and glutamine), aspartic acid, histidine, proline, and hydroxyproline were the most active. Both yeast extract and acid-hydrolysed casein also improved growth on the basal medium containing glucose and ammonium salts (quantities as in Table 1) in place of creatine. In the presence of yeast extract the further addition of either glucose or of ammonium salt to the creatine medium led to a considerable increase in total growth (Table 4).

pH range. Growth of the organism was remarkably independent of hydrogen-ion concentration, and was almost equally good between pH values of 5.4 and 9.0 with an ill-defined optimum in the region of pH 8; pH 7.0 (which was also about the optimum for creatine destruction) was chosen for all subsequent work.

The importance of other ions was also investigated briefly. A fairly high concentration of NaCl was found to be obligatory; below 0.1 % (w/v) growth was suboptimal, and above 2 % the salt was inhibitory. Traces of Ca, Mg, Mn and Fe were essential.

Temperature. A temperature of 30° was near the optimum for growth. Incubation at 25° resulted in a slight decrease; raising the temperature to 37° decreased growth at 24 hr. to about one-half of that at 30°.

Aeration. Roux bottles incubated horizontally provided the largest crops. Growth after 24 hr. was decreased considerably when the bottles were kept in the vertical position, or even when they were sloped so as to present half the normal surface area to the atmosphere.

An attempt was made to increase the crop by cultivating in medium continuously agitated by a magnetic stirrer. Although this procedure must have resulted in increased aeration, growth after 24 hr. was only one-third of that in undisturbed Roux bottles. A similar decrease was observed in Roux bottles which were shaken by hand every few hours. To test whether a raised oxygen tension might be inhibitory, a Roux bottle was filled with oxygen after inoculation; growth at 24 hr., however, was as good as in a bottle exposed to air in the usual way.

Substrates for growth. In testing the ability of the organism to use a variety of single substances for growth these were added to the basal medium without yeast extract to give a concentration of 0.3 % (w/v); when pairs of substances were tested each was present at 0.25 %. Failure to grow was recorded when no visible growth occurred after 3 to 4 days. A number of nitrogenous organic compounds were tested for their ability to support growth (Table 1); non-nitrogenous organic compounds were tested in the presence of ammonium

salts. Of most interest was the limited ability to grow on guanidine derivatives; of those tested only creatine, arginine and the amine of arginine, agmatine, supported full growth. There was a limited ability to use guanidine itself, but none to use creatinine or glycoxyamine. Urea was used as a nitrogen source (in the presence of glucose as a carbon source), and sarcosine as source of both nitrogen and carbon.

Table 1. *Substances tested for their ability to support growth of Pseudomonas ovalis Chester as source of C and N*

Single compounds at 0.3 % (w/v) in the basal medium; NH_4^+ salt and carbon source at 0.25 % each. 0=no growth up to 5 days; + + + + = abundant growth, etc.

(a) Guanidine derivatives			
Creatine	+ + + +	Methylguanidine	0
Creatinine	0	Dimethylguanidine	0
Glycoxyamine	0	Ethylguanidine	0
Arginine	+ + +	Tetramethylenediguanidine	0
Agmatine	+ + + +	Decamethylenediguanidine	0
Guanidine	+		
(b) Possible primary breakdown products of creatine			
Sarcosine	+ + + +	Urea + glycine	0
Glycine	0	Urea + glucose	+ + + +
Urea	0		
(c) Amino acids and miscellaneous compounds			
Alanine	±	Asparagine	+
Serine	±	NH_4 + glucose	+ + + +
Aspartate	0	NH_4 + acetate	++
Glutamate	0	NH_4 + citrate	+
Methionine	0	Choline	+ + +
Tryptophan	0	Indole	0

Action of suspensions on creatine

When suspensions of organisms harvested from the creatine medium were incubated aerobically with creatine, the substrate disappeared at a uniform rate until all had been removed (Fig. 2). Organisms destroyed their own (dry) weight in 1–2 hr.

The disappearance of creatine was accompanied by uptake of O_2 and by formation of CO_2 , NH_3 and urea. In the absence of any added substrate suspensions of organisms had a Q_{O_2} of 20–30; this endogenous uptake was always subtracted from the values observed in the presence of substrate to give the recorded values. In the presence of creatine the corrected Q_{O_2} was about 100 and O_2 uptake continued at a regular rate during the removal of creatine. At, or slightly after, the time when the creatine had completely disappeared the rate of O_2 consumption abruptly fell to a much lower level. Yet even when incubation was continued for many hours after the 'break' in the curve, the rate of O_2 consumption never fell quite to the value without substrate (Fig. 2).

At the time of the 'break' in the O_2 uptake curve about 1.4 mole O_2 /mole creatine (limits 1.3–1.5 mole) were taken up. Production of CO_2 followed a

course very similar to that of O_2 uptake (Fig. 2). At the 'break' about 1.5 mole CO_2 /mole creatine were formed.

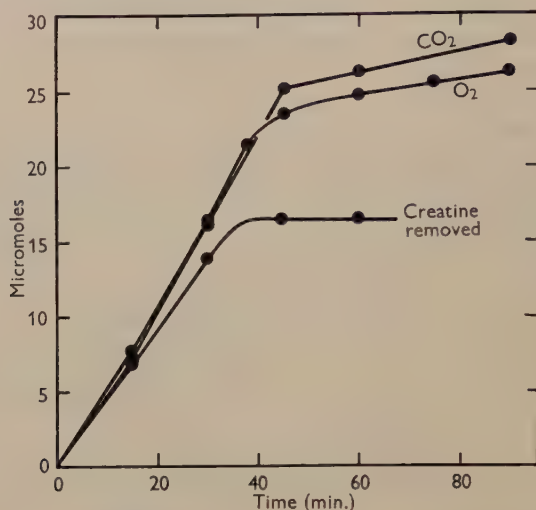


Fig. 2. Uptake of O_2 and evolution of CO_2 in relation to creatine disappearance. Organisms (equiv. to 5 mg. dry wt.) in 0.08M-phosphate buffer (pH 7.8) with $16.7 \mu\text{mole}$ creatine; total volume 2.5 ml. Incubated at 30° in air.

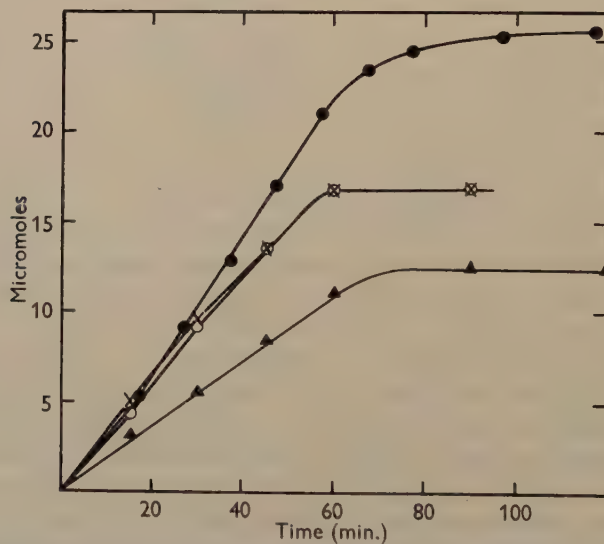


Fig. 3. Creatine removal (x), O_2 uptake (●), urea (O) and NH_3 (▲) formation by suspensions of *Pseudomonas ovalis*. Organisms 6 mg. (dry wt.); other additions as in Fig. 2.

About 0.7 mole NH_3 (limits 0.65–0.75) was formed (Fig. 3). On two occasions ammonia was estimated as volatile base in the Markham apparatus and by its colour production with Nessler's reagent. At the concentrations used

methylamine, a possible product of creatine breakdown, behaved as ammonia on steam distillation but gave no colour with Nessler's reagent. Since the values given by the two methods were in excellent agreement it was concluded that no methylamine was being produced. At all stages of the reaction 1 mole urea/mole creatine was formed (Fig. 3).

Amino acids were sought by paper partition chromatography, fatty acids by distillation with phosphoric acid in the Markham apparatus, and formaldehyde by its colour reaction with chromotropic acid. None of these compounds was detected in the reaction mixture.

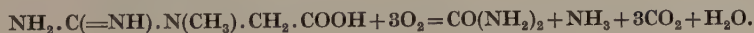
Oxidative assimilation. The degradation of creatine to CO_2 , ammonia and urea required in theory the appearance of 3 mole CO_2 and one each of the other two products, together with the uptake of 3 mole O_2 . The considerable discrepancies between the observed values (with the exception of urea) and the theoretical suggested either that there were undetected products or that some of the creatine might be undergoing oxidative assimilation (Clifton, 1946). To test the latter possibility organisms were incubated with creatine in the presence of sodium azide or 2:4-dinitrophenol. Different batches of organisms varied considerably in their sensitivity to azide and the optimal concentration varied by as much as a factor of three from one suspension to another. In the presence of that concentration of azide (*c.* 10^{-3}M) which had the most marked effect the O_2 consumption at the 'break' was increased only by a factor of about 1.2 (Table 2); there was a similar proportionate increase in CO_2 and NH_3 production. It is clear that it is not possible to account for the missing creatine by oxidative assimilation unless this is considerably greater than it was possible to demonstrate.

Table 2. *Effect of azide on the oxidation of creatine by Pseudomonas ovalis Chester*

Organisms (equiv. to 4 mg. dry wt.) were incubated in 0.067M-phosphate buffer (pH 7) with $16.7\text{ }\mu\text{mole}$ creatine in presence or absence of sodium azide ($2\text{ }\mu\text{mole}$); total volume 3 ml. The reaction was stopped after 100 min.; the 'break' in O_2 uptake occurred after 60 min. in absence of azide and after 90 min. in its presence. All creatine had been removed in both cases.

	Moles/mole creatine removed		
	Azide absent	Azide present	Theory*
O_2 taken up	1.55	1.95	3.0
CO_2 formed	1.74	2.01	3.0
NH_3 formed	0.70	0.81	1.0
Urea formed	0.99	1.01	1.0

* For complete oxidation according to



Several attempts were made to confirm this 'uncoupling' effect with dinitrophenol. On only one occasion was the O_2 uptake increased (to 2.0 mole); at all other times dinitrophenol was without effect.

Kinetics of creatine destruction

Concentration of organisms. Below a concentration of *c.* 3 mg. dry wt. organisms/ml. the rate of creatine removal and O_2 uptake was directly proportional to the concentration of organisms. Above this concentration O_2 consumption increased only slowly and was never more than 1000 μ l./hr. At high suspension densities the factor limiting rate of O_2 uptake appeared to be the rate at which O_2 diffused into the reaction mixture or into the organisms. When the flasks were filled with O_2 instead of with air a far greater rate of uptake was attained (Fig. 4).

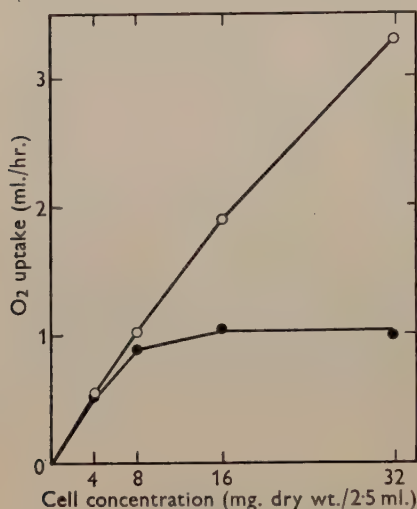


Fig. 4

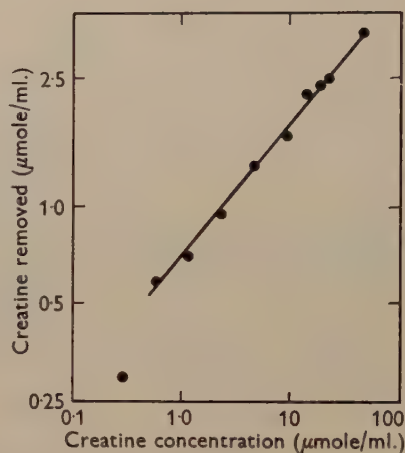


Fig. 5

Fig. 4. Effect of O_2 tension on activity of suspensions of *Pseudomonas ovalis*. Organisms (different concentrations) with other additions as in Fig. 2. Shaken at 30° in air (●) or O_2 (○). Not corrected for endogenous O_2 uptake.

Fig. 5. Influence of initial creatine concentration upon rate of creatine removal by *Pseudomonas ovalis*. Different amounts of creatine were incubated with suspensions of organisms equivalent to 1.76 mg. dry wt. in 4 ml. 0.05M-phosphate buffer (pH 7) for 50 min. at 30° .

Creatine concentration. Oxygen uptake reached a maximum rate when the creatine concentration was about 10^{-2} M. The rate of creatine disappearance, however, continued to increase up to at least 4.7×10^{-2} M, the highest concentration tested. Over almost a 100-fold range of concentration there was a linear relationship between the logarithm of the initial creatine concentration and the logarithm of its rate of destruction (Fig. 5).

pH range. The optimum pH for destruction of creatine in 0.05M-phosphate buffer was in the region of 8.0. There was no sharp peak in the pH-activity curve (Fig. 6). In spite of the slightly decreased activity in the presence of 0.05M-borate a much better-defined optimum than with phosphate was found at pH 8.25 (Fig. 6). Where comparison could be made the rate of creatine

removal was even less in the presence of veronal; for instance at pH 7·8 the rate was one-third of the rate in phosphate. As the pH value was increased to the end of the veronal range (about pH 9·4) the rate rose steadily to 70 % of that at the phosphate optimum.

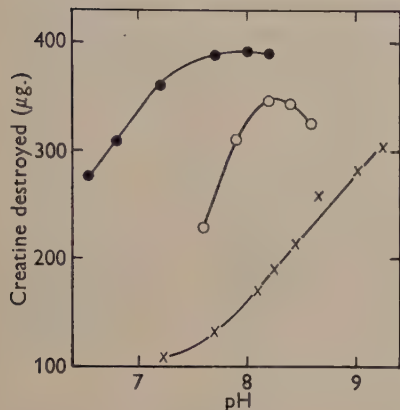


Fig. 6

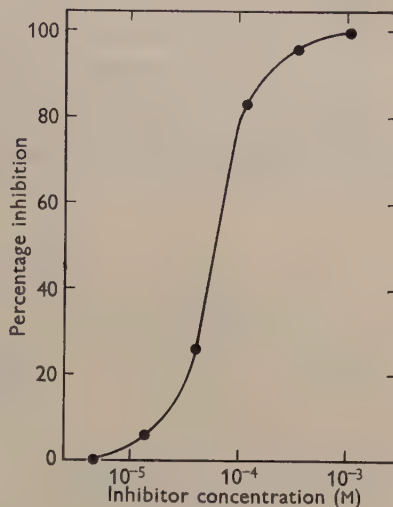


Fig. 7

Fig. 6. Effect of pH and of nature of buffer on creatine destruction of *Pseudomonas ovalis*. Four ml. of 0·05 M-phosphate (●), borate (○), or veronal (×) buffer containing the equivalent of 1·08 mg. dry wt. organisms and 1·0 mg. creatine; incubated at 30° for 45 min.

Fig. 7. Inhibition by *p*-chloromercuribenzoate of creatine removal by *Pseudomonas ovalis*. Organisms (equivalent to 1·35 mg. dry wt.) in 4 ml. 0·05 M-phosphate buffer (pH 7) with 1·0 mg. creatine; incubated 1 hr. at 30°.

Inhibitors. *p*-Chloromercuribenzoic acid (as the sodium salt) completely inhibited destruction of creatine. With a suspension equivalent to 0·2 mg. dry wt. organisms/ml. 50 % inhibition was induced by $6 \cdot 6 \times 10^{-5}$ M-*p*-chloromercuribenzoate (Fig. 7). In a further experiment, with a suspension of organisms ten times more concentrated, O₂ uptake was inhibited 50 % by a $4 \cdot 5 \times 10^{-5}$ M-*p*-chloromercuribenzoate.

During investigation of the effect of pH value upon the reaction it was discovered that acetate caused a powerful inhibition (Fig. 8). Other fatty acids were also inhibitory, the order of decreasing activity being acetate, propionate, butyrate, formate; these tests were made at pH 6. Creatine removal was inhibited 50 % by acetate at 5×10^{-3} M, propionate at 7×10^{-3} M and butyrate at 2×10^{-2} M. Formate was much less effective; at 5×10^{-2} M (the highest concentration tested) it produced only 25 % inhibition.

Inhibition by acetate was completely independent of creatine concentration. A concentration of acetate expected to cause about 50 % inhibition was added to a series of flasks, in which the creatine concentration was varied over a 64-fold range. The degree of inhibition varied only between 50 and 65 %.

Loss of activity on storage and washing. Harvested organisms lost some activity either during storage at 0° (especially when exposed to air) or on further washing with phosphate buffer (Table 3). Activity was partly or wholly restored by Difco yeast extract (1 mg./ml.) or by DL-cystine (125 mg./ml.).

Table 3. *Loss of creatine-destroying activity induced by washing Pseudomonas ovalis with phosphate buffer, and stimulation by yeast extract*

Organisms (equivalent to 0.55 mg. dry wt.) were incubated for 1 hr. at 30° in a volume of 4 ml. at pH 7.8 with 1000 μ g. creatine, with or without yeast extract (1 mg./ml.). Organisms from normal suspensions (once washed) were centrifuged and washed repeatedly with 0.02M-phosphate buffer pH 7; samples were taken after each washing.

Number of extra washes	Suspension	
	No yeast extract	With yeast extract
	Creatine removed (μ g.)	
0	473	552
1	308	496
2	200	400
3	185	420

Adaptive nature of creatine oxidation

Only organisms grown with creatine as main carbon and nitrogen source possessed full ability immediately to remove creatine. Organisms grown with either arginine or with glucose + ammonia removed no creatine during an incubation period (1 hr.) when creatine-grown organisms had removed the whole of the substrate. When incubation was continued beyond 1 hr. organisms grown in the absence of creatine began slowly to oxidize this compound and continued at an increasing rate (Fig. 9).

As described earlier, the addition of either glucose, or of ammonium salt, or of both, considerably increased the total amount of growth in the creatine medium. Organisms grown in this way, however, showed a loss of intrinsic activity which more than offset the increased growth (Table 4). Organisms were also grown on the usual creatine medium solidified with 3% (w/v) agar; growth was then about 70% better than in the liquid medium, but activity/amount of organism was decreased almost proportionately.

Specificity of action of suspensions

Organisms were grown on media containing creatine, arginine, or glucose + ammonium salt and their activity towards a number of guanidine derivatives and related compounds was tested. Some of the results of such experiments, in which the criterion of activity was the disappearance of substrate, are summarized in Table 5. Tests on the specificity of creatine-grown organisms were extended to include several further compounds which develop a colour with diacetyl and α -naphthol in alkaline solution. None of the compounds tested was attacked (Table 6). In addition, manometric experiments showed that organisms grown in a creatine medium were unable to oxidize creatine

Table 4. *Effect upon growth of Pseudomonas ovalis and upon creatine-oxidising activity of supplementing the creatine medium with ammonium salts and glucose*

Cultures of *P. ovalis* were incubated in Roux bottles at 30° for 24 hr.; the normal creatine-containing medium had the supplements stated. Organisms (equivalent to 8–16 mg. dry wt.) incubated in 0.08 M-phosphate buffer (pH 7.8) with 16.7 μ mole creatine. Endogenous O₂ uptake not subtracted.

Supplement to growth medium	Growth (mg. dry wt./100 ml.)	Q _{O₂} with creatine
(1) Nil	38	124
(2) (NH ₄) ₂ SO ₄ , 0.25 % (w/v) + NH ₄ Cl, 0.25 % (w/v)	51	86
(3) Glucose, 0.5 % (w/v)	69	50
(4) (2) + (3)	83	33

Table 5. *Influence of the growth substrate upon the relative ability of suspensions of Pseudomonas ovalis to remove various substrates*

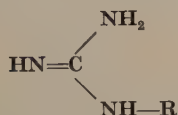
Suspensions of organisms (equivalent to 1.0–1.3 mg. dry wt.) harvested from the basal medium with indicated supplements and incubated for 1 hr. with 1.0 mg. creatine or an equivalent weight of the other substrates.

Substrate	Relative rate* of removal of substrate by organisms grown on		
	Creatine	Arginine	NH ₄ ⁺ + glucose
Creatine	100	0	0
Arginine	10	100	0
Agmatine	30	50	0
Glycocyamine	0	0	0
Creatinine	0	0	0

* Rate of removal of creatine by creatine-grown organisms = 100.

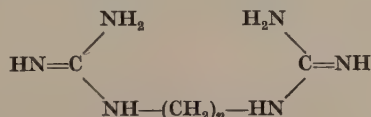
Table 6. *Guanidino compounds not attacked by creatine-grown Pseudomonas ovalis*

(a) Monoguanidino derivatives



No.	R =
1	—H
2	—CH ₃
3	—CH ₂ —CH ₃
4	—(CH ₂) ₂ —CH ₃
5	—CH=(CH ₃) ₂
6	—(CH ₂) ₃ —CH ₃
7	—CH ₂ —CH=(CH ₃) ₂
8	—(CH ₂) ₃ —N=(C ₂ H ₅) ₂
9	—(CH ₂) ₁₁ —CH ₃
10	—(CH ₂) ₁₃ —CH ₃
11	—(CH ₂) ₁₅ —CH ₃
12	—(CH ₂) ₁₇ —CH ₃
13	—2-benziminazole

(b) Diguanidino derivatives



No.	n =
14	4 (arcaine)
15	5
16	6
17	8
18	10 (synthalin)
19	12 (synthalin B)

phosphate, dimethylguanidine, hydantoic acid or urea oxalate. Absence of O_2 uptake confirmed their inability to oxidize guanidine, methylguanidine or creatinine.

When creatine-grown organisms were incubated in the usual way with glyco-cyamine, O_2 consumption was about 50 % higher than the endogenous value; i.e. there was a Q_{O_2} of about 10 apparently due to the oxidation of glyco-cyamine. On the other hand, colorimetric estimation showed no loss of glyco-cyamine, and even after 5 hr. of incubation, no urea or ammonia was detected

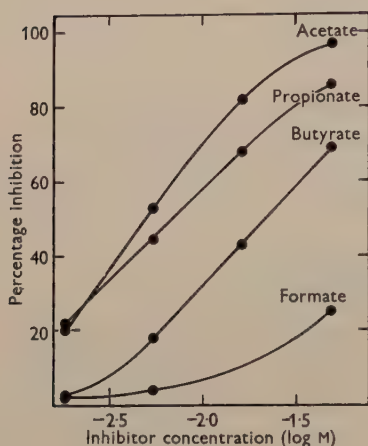


Fig. 8

Fig. 8. Inhibition by fatty acids of *Pseudomonas ovalis*. Organisms (equivalent to 1.17 mg. dry wt.) incubated as in Fig. 7 except that buffer was pH 6. Acids brought to pH 6 before addition.

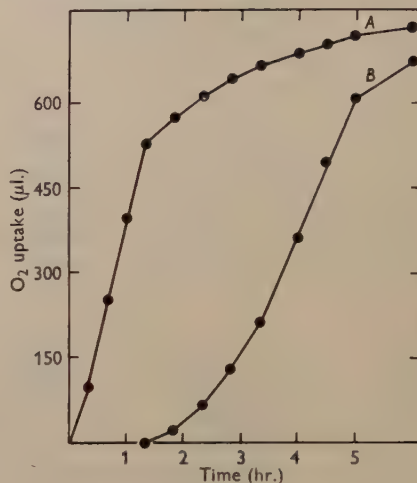


Fig. 9

Fig. 9. Effect of growth substrate upon oxidation of creatine by suspensions of *Pseudomonas ovalis*. Organisms (equivalent to 4.5 mg. dry wt.) grown on medium containing creatine (A) or glucose and ammonium salts (B) and incubated in 0.08 M-phosphate buffer (pH 7.8) with 16.7 μ mole creatine.

in the reaction mixture. It was thought just possible that glyco-cyamine was being converted to guanidine (by oxidation of the acetic acid side-chain) or to some other guanidino compound giving a similar colour reaction; yet paper chromatography gave no evidence of this. It was concluded that such suspensions of organisms were unable to oxidize glyco-cyamine.

DISCUSSION

The micro-organism isolated in the present work appears to produce an adaptive system of enzymes which enable it to grow with creatine as main source of carbon and nitrogen. It differs from other organisms having this property in its high degree of specificity. In particular it does not attack creatinine; it is unable to grow on a medium containing creatinine as main organic constituent,

and washed organisms do not destroy creatinine. The only other guanidine derivatives which are attacked by creatine-grown organisms are arginine and agmatine, which are destroyed relatively slowly. The mechanisms of degradation of these two compounds are probably different from that of creatine, since organisms grown on an arginine medium are unable to metabolize creatine. This degree of specificity provides the basis for a method for the selective destruction of creatine in a mixture which also contains creatinine or other guanidine derivatives. It has been exploited by Ennor & Stocken (1953) in determining the urinary excretion of creatine.

Of the products of the reaction in which creatine was destroyed only urea was found in theoretical yield. The formation of 1 mole urea from 1 mole guanidine derivative appears to be a rather constant finding in other cases where this point has been investigated (Dubos & Miller, 1937; Kopper & Beard, 1947; Krebs & Eggleston, 1939; Roche *et al.* 1948). An exception to this was found with arginine; Hills (1940) showed that certain Gram-positive cocci contain an enzyme, arginine dihydrolase, which hydrolyses arginine directly to ornithine, NH_3 and CO_2 without the intermediate formation of urea. Roche, Lacombe & Girard (1950) studied the action on arginine of growing cultures of our strain of *Pseudomonas ovalis* Chester, and concluded that it also produces an arginine dihydrolase.

Further work on the reactions involved in the degradation of creatine by this organism is described in the following paper (Appleyard & Woods, 1956).

This work was carried out during the tenure by one of us (G.A.) of a Medical Research Council Scholarship.

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The Pathway of Creatine Catabolism by *Pseudomonas ovalis*

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SUMMARY: Creatine is oxidized by suspensions of a strain of *Pseudomonas ovalis* Chester harvested from media containing creatine as main source of carbon and nitrogen. A possible pathway of degradation of at least part of the creatine is: (a) hydrolysis of creatine to sarcosine and urea, (b) oxidation of sarcosine to glycine and formaldehyde, (c) oxidation of the latter products to CO₂ and NH₃ with considerable concurrent oxidative assimilation.

Soluble enzyme preparations catalysing stage (a) were obtained by aqueous extraction of either acetone-dried or toluene-treated organisms. The enzyme for stage (b) was present in the insoluble residue from the latter organisms.

The literature concerning micro-organisms able to grow with creatine or creatinine as sole source of carbon and nitrogen has been surveyed by Nimmo-Smith & Appleyard (1956). The ability of such organisms to grow on related compounds or derivatives (or to metabolize them in cell suspension) may give indirect evidence as to possible pathways of creatine metabolism. A compound which is not utilized is unlikely (unless unable to enter the organism) to be an intermediate; conversely, a compound which is rapidly attacked may be, but is not necessarily, on the pathway. On these grounds possible intermediates in the degradation of creatine by various micro-organisms are creatinine, sarcosine, glycoeyamine and methylguanidine (Table 1); it may be noted that with no organism is sarcosine excluded. Glycoeyamine and methylguanidine appear as 'possible' with some organisms but 'unlikely' with others.

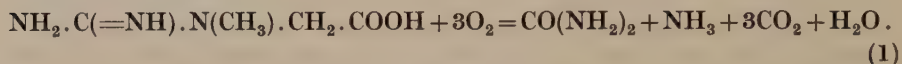
Table 1. *Specificity of organisms able to grow on creatine*

Organism	Related compounds which		Reference
	Support growth	Do not support growth	
'HR'	—	Glycoeyamine, methylguanidine	(1)
'NC' (<i>Corynebacterium creatinovorans</i>)	Creatinine, methylguanidine, glycoeyamine, sarcosine	—	(1)
A bacterium	Creatinine	—	(2)
A mould	—	Glycoeyamine	(3)
<i>Pseudomonas ovalis</i>	Glycoeyamine, sarcosine	—	(4)
<i>P. aeruginosa</i>	Sarcosine	Glycoeyamine	(5)

References: (1) Dubos & Miller (1937); (2) Beard (1943); (3) Beard (1944); (4) Roche, Girard, Lacombe & Mourgue (1948); (5) Kopper (1947).

A more extensive study of creatinine and creatine catabolism has been made with a strain of *Pseudomonas aeruginosa* which grows well on a medium containing either of these substances (Kopper & Beard, 1947; Kopper, 1948). Creatinine was first converted to creatine; since suspensions of organisms oxidized sarcosine the next step was thought to be the hydrolysis of creatine to sarcosine and urea, but no direct evidence for this reaction was found. The breakdown of sarcosine by this organism was also studied (Kopper & Robin, 1950; Kopper, 1950); this work will be discussed later.

The organism used in the present work was a strain of *Pseudomonas ovalis* Chester isolated by Nimmo-Smith (1949). It degrades creatine, but unlike the other organisms referred to above, does not attack creatinine. It was shown in the preceding paper (Nimmo-Smith & Appleyard, 1956) that suspensions of organisms harvested from a medium containing creatine oxidized this substance with overall formation of urea, NH_3 and CO_2 . Quantitatively the values for O_2 , CO_2 and NH_3 were only 45, 50 and 70 %, respectively, of those expected for the reaction (1) below, but there was evidence that these low values were due, at least in part, to oxidative assimilation; the yield of urea was theoretical:



A more detailed study of the course of this reaction with whole organisms and enzyme preparations is the subject of the present paper. Some of the results were briefly reported by Appleyard (1951).

METHODS

Organisms

Pseudomonas ovalis Chester (*National Collection of Type Cultures*, no. 7914). The isolation and maintenance of this organism and the preparation of suspensions after growth on a medium containing creatine are described by Nimmo-Smith & Appleyard (1956).

Organism 'S'. A contaminant found growing at refrigerator temperature in a solution of sarcosine (0.033M) was isolated; it appeared to be a pseudomonad but was not further characterized. The organism grew moderately on a medium similar to that used for *Pseudomonas ovalis*, but containing sarcosine (5 g./l.) in place of creatine; there was no growth on creatine. Suspensions of this organism, prepared as above, were used for the assay of sarcosine.

Preparations of organisms

Acetone-dried organisms. A suspension of organisms (50 mg. dry wt./ml.) was mixed with cold acetone (10 vol.) and centrifuged. The deposit was washed three times on the centrifuge with 4 vol. quantities of acetone and finally stored *in vacuo* at 4°. This preparation hydrolysed creatine to sarcosine and urea and retained its activity for more than three months.

At least 90 % of the enzyme was obtained in solution by suspending the dried organisms in 0.02M-phosphate buffer (pH 7) and removing the insoluble residue by centrifuging. This extract was used for most experiments.

Toluene-treated organisms. A suspension of organisms (10 ml.; 10 mg. dry wt./ml.) was shaken with toluene (1 ml.) for 10 min. After centrifuging, the organisms were washed once with water (10 ml.) and finally suspended in 10 ml. water. Activity was retained for at least 24 hr., though the preparation was normally used immediately.

Crushed organisms. The principle of Curran & Evans (1942) was used. Cylindrical bottles (30 ml.), containing 8 ml. organism suspension (20 mg. dry wt./ml.), 10 g. glass balls (diameter 0.2 mm.; Ballotini no. 13, Chance Bros., Smethwick) and 0.1 ml. tributyl citrate (anti-foam), were filled with N₂ and shaken vertically at 400 oscillations/min. for 1 hr. at approximately 10°. The glass balls were removed by filtration.

Estimations

Uptake of O₂ and formation of CO₂, NH₃ and urea were measured as described by Nimmo-Smith & Appleyard (1956).

Creatine. The method of Eggleton, Elsdon & Gough (1943), as applied by Nimmo-Smith & Appleyard (1956), was used. Sarcosine was found slightly to decrease the colour given by creatine, the percentage inhibition being proportional to the sarcosine concentration. In experiments in which sarcosine accumulated in the reaction mixture it was necessary therefore to apply a correction; this never exceeded 7 %.

Sarcosine. Suspensions of organism 'S' oxidize sarcosine, glycine, formaldehyde and acetate, but not creatine or urea. Approximately 1.6 mole O₂/mole sarcosine were taken up and the uptake was strictly proportional to the amount of sarcosine added over the range tested (1–8 μ mole) (Fig. 1). It was possible to estimate sarcosine with an accuracy of ± 5 % in reaction mixtures known not to contain other oxidizable substrates; this method was applied in experiments in which creatine was hydrolysed to sarcosine and urea by acetone-dried organisms or by whole organisms anaerobically. Manometer vessels were set up containing CO₂ absorbers, 1 ml. bacterial suspension (3 mg. dry wt./ml.), 1 ml. phosphate buffer (0.2M, pH 7.8) and 1 ml. of either water (control), standard sarcosine or experimental sample (containing 2–8 μ mole sarcosine). O₂ uptake was measured at 30° until it fell to the endogenous value (usually in 1–2 hr.).

Glycine. A microbiological assay with *Leuconostoc mesenteroides* P60 (American Type Culture Collection, no. 8042) was used. The technique was modified from that of Lascelles, Cross & Woods (1954) for assay of serine only in that serine (10⁻³M) was added to the medium and glycine omitted.

Formaldehyde. This was detected and estimated with an accuracy of ± 10 % by its colour reaction with 1,8-dihydroxynaphthalene-3,6-disulphonic acid (chromotropic acid; MacFadyen, 1945). Methanol and formic acid do not react.

Chromatography

Descending chromatograms were developed on Whatman no. 4 paper with either 'phenol' (a saturated solution of water in A.R. phenol) or 'butanol/acetic acid' (the upper layer of a mixture of *n*-butanol, glacial acetic acid and water in the proportion of 4:1:5). The detecting agent was ninhydrin; sarcosine gives a positive reaction but the colour fades more rapidly than with most amino acids.

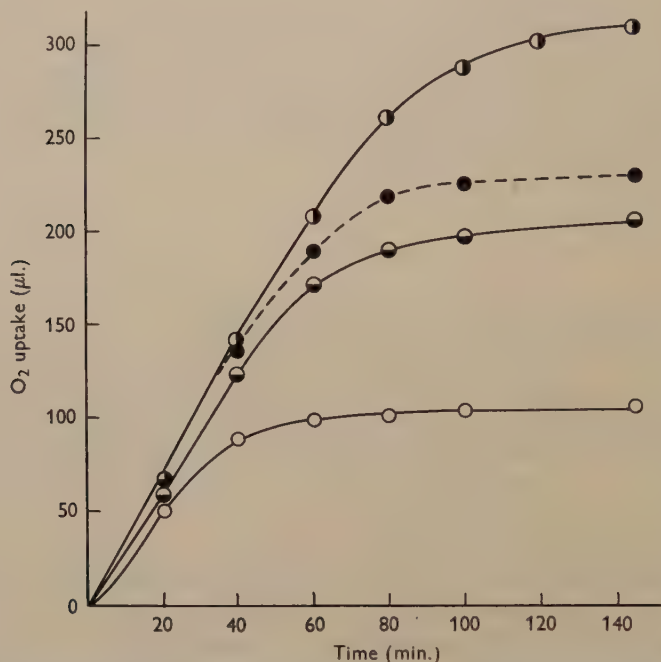


Fig. 1. Course of oxidation of sarcosine by organism 'S'. Organisms (3 mg. dry wt.) incubated in 0.067 M-phosphate buffer pH 7.8 with 2.8 (○), 5.6 (◐) and 8.3 (●) μmole sarcosine or with a sample of an experimental product (●).

Glycine was detected and separated from other reaction products by chromatography on Dowex-50 resin (Moore & Stein, 1951, as applied by Lascelles & Woods, 1954).

Experiments with suspensions of organisms

In general, reaction mixtures were incubated at 30° in Warburg manometers, either aerobically or anaerobically (in N₂). The vessels contained cell suspension or enzyme preparation (1 ml.) and 0.2 M-phosphate buffer pH 7.8 (1 ml.) in the main compartment. Substrates (0.5 ml.; 0.033 M or as stated) were added from a side-bulb after equilibration. Reactions were stopped by the addition of 2N-H₂SO₄ (0.1 ml.) or by heating to 100° for 5 min. The organisms were removed by centrifuging and estimations carried out with samples of the clear supernatant fluid. To avoid any loss of NH₃ during manipulations a phosphate buffer of pH 7 was used in the reaction mixture when NH₃ was to be estimated.

RESULTS

Hydrolysis of creatine to sarcosine and urea

Anaerobic action of whole organisms. Creatine disappeared slowly when incubated anaerobically with suspensions of *Pseudomonas ovalis* Chester. Urea was detected in the reaction products by the production of NH_3 when further incubated with urease. Chromatography on paper showed the presence of a single ninhydrin-positive substance with an R_F of 0.79 in 'phenol' and 0.17 in 'butanol/acetic acid'. The same R_F values were given by pure sarcosine and by samples from experiments in which sarcosine and urea had been incubated with suspensions. Samples from experiments in which organisms were incubated with creatine + sarcosine gave only one ninhydrin-positive spot corresponding to sarcosine.

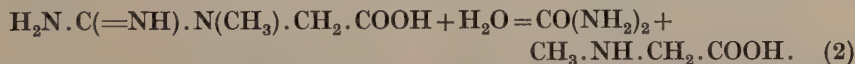
Samples of the products also took up O_2 when incubated with suspensions of organism 'S'; the time course of the oxidation was similar to that of sarcosine (Fig. 1).

Table 2. *Hydrolysis of creatine by suspensions of Pseudomonas ovalis Chester anaerobically and by creatinase preparations*

Creatine (67.1 μmole) incubated in 0.067 M-phosphate buffer pH 7.8 with either (a) suspension of whole organisms (10 mg. dry wt.) in an atmosphere of N_2 , or (b) creatinase (extract from 16 mg. acetone-dried organisms) in air. Total volume 6 ml. Controls incubated without creatine.

	μmole (less control values) with					
	Whole organisms after (hr.)			Creatinase after (hr.)		
	1	2	3	1	2	3
Creatine (removed)	18.4	30.1	38.1	17.2	28.3	34.1
Sarcosine (formed)	16.3	29.5	36.5	16.5	26.6	33.7
Urea (formed)	15.7	29.1	37.4	15.4	27.3	33.2

Quantitative experiments showed the production of 1 mole each of sarcosine and urea for each mole of creatine decomposed (Table 2); no CO_2 or NH_3 was formed. The anaerobic reaction catalysed by the whole organisms is therefore:



The rate of anaerobic removal of creatine at the final concentration normally used ($6 \times 10^{-3} \text{M}$) was only about 20 % of the aerobic rate (Fig. 2). The addition of an equal quantity of sarcosine (which accumulates anaerobically) depressed the anaerobic rate by only 10 %. The difference between the aerobic and anaerobic rates decreased with increasing concentration of creatine until, at $6 \times 10^{-2} \text{M}$ (an almost saturated solution), they became almost equal (Fig. 2).

Action of acetone-dried organisms. Neither suspensions of acetone-dried organisms nor the soluble fraction extracted with phosphate buffer took up O_2 in the presence of creatine. Both preparations catalysed the same reaction as whole organisms under anaerobic conditions, i.e. the quantitative hydrolysis

of creatine to sarcosine and urea (Table 2). Suspensions of the dried organisms did not attack either sarcosine or urea, nor were they able to condense these two substances with formation of creatine. There was no action on creatine phosphate.

The enzyme which hydrolyses creatine to urea and sarcosine will be referred to as creatinase. The soluble preparation extracted from acetone-dried organisms was stable, though its activity was somewhat variable with different

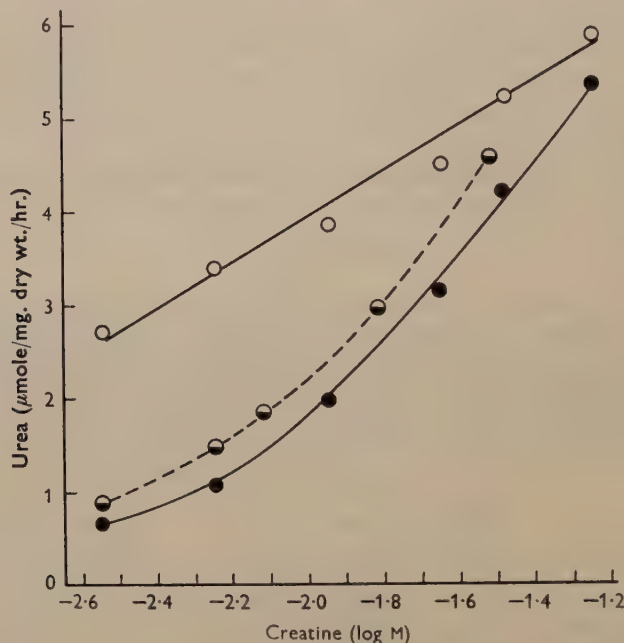


Fig. 2. Effect of substrate concentration on the hydrolysis of creatine by suspensions of *Pseudomonas ovalis*: aerobically (○) or anaerobically in N_2 (●) and by a creatinase preparation aerobically (◐). Hydrolysis followed by estimating urea produced after incubating whole organisms (5 mg. dry wt./ml.) or creatinase (extract from 6 mg. acetone-dried organisms/ml.) in 0.067 M-phosphate buffer pH 7.8 with the concentrations of creatine shown.

batches of original whole organisms. Under optimal conditions of pH (7.8) and temperature (30°), and with an initial creatine concentration of 6×10^{-3} M, the rate of creatine removal was usually in the range 0.35–0.75 μ mole/mg. dry wt. organisms/hr. This could be increased by raising the creatine concentration (Fig. 2); in a single experiment the K_m of creatinase was found to be 2.3×10^{-2} M. Over the range of creatine concentration tested the enzyme preparation was never more than 30% more active than the equivalent amount of whole cells acting anaerobically.

The effect of certain enzyme inhibitors was tested (Table 3). The inhibition of creatinase by *p*-chloromercuribenzoate (4×10^{-5} M) was almost completely overcome by four times this concentration of DL-cysteine.

Creatinase was partially purified by precipitation with $(\text{NH}_4)_2\text{SO}_4$; the fraction collected at 40–50 % saturation contained the bulk of the activity. Repeated precipitation at 50 % saturation caused a progressive, though variable, loss of activity amounting usually to about 70 % after three precipitations.

Table 3. *The effect of inhibitors on the creatinase of Pseudomonas ovalis Chester*

Extract from 8 mg. acetone-dried organisms incubated at a final volume of 3 ml. in 0.067 M-phosphate buffer pH 7.8 (unless otherwise stated) with creatine ($16.8 \mu\text{mole}$) and inhibitor; controls without inhibitor. Disappearance of creatine estimated.

Inhibitor	Time incubated (hr.)	Final concn. of inhibitor (M)	Percentage inhibition of creatine removal
Borate, pH 7.8	1.0	5×10^{-2}	0
Borate, pH 9.1	1.0	5×10^{-2}	80
Azide	2.0	2×10^{-2}	18
Cyanide	2.0	10^{-2}	0
Iodoacetate	1.5	10^{-2}	20
p-Chloromercuribenzoate	2.0	4×10^{-5}	100

The activity was almost completely restored by the addition of extracts from whole or acetone-dried organisms prepared by heating suspensions (30 mg. dry wt./ml.) to 100° for 5 min. and centrifuging (Fig. 3). Reducing compounds such as glutathione, cysteine, mercaptoacetic acid and ascorbic acid also reactivated the enzyme either partially or completely; glutathione was the most effective, giving 90 % reactivation at about 10^{-3}M (Fig. 3). These

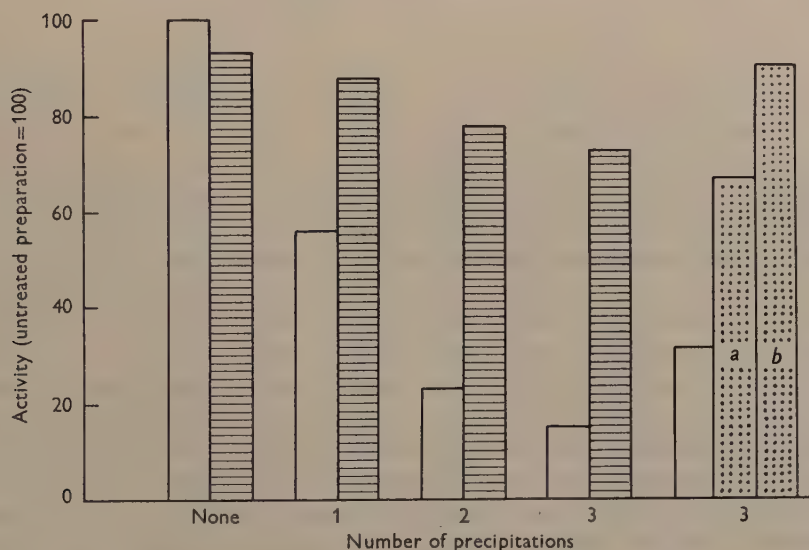


Fig. 3. Loss of creatinase activity on repeated precipitation by half-saturation with ammonium sulphate. Enzyme derived from 8 mg. acetone-dried *Pseudomonas ovalis* incubated in 0.067 M-phosphate buffer pH 7.8 with $16.8 \mu\text{mole}$ creatine; total volume 3 ml. Supplements: \square , none; \equiv , supernatant from 30 mg. heated acetone-dried organisms; \equiv , reduced glutathione (a) $40 \mu\text{mole}$, (b) 5 mmole.

results, together with those above for *p*-chloromercuribenzoate, suggest strongly that creatinase has free thiol groups.

Creatinase could not be extracted from *Pseudomonas ovalis* Chester harvested from a medium containing glucose and ammonium salts in place of creatine. This confirms the previous conclusion (Nimmo-Smith & Appleyard, 1956) that the enzyme systems metabolizing creatine are adaptive.

Action of crushed organisms. No oxygen was taken up by these preparations in the presence of creatine but, under anaerobic conditions, one molecular equivalent of urea was formed: no tests were made for sarcosine in this case. The enzyme was again soluble and present in the supernatant fluid after centrifuging the crushed material. The initial rate of removal of creatine was about 40 % greater than that of the uncrushed organisms acting anaerobically. Since both whole crush and the supernatant fluid lost activity rapidly on shaking in air, this type of creatinase preparation was not further used.

Action of toluene-treated organisms. The supernatant fluid obtained by centrifuging these preparations also contained an enzyme which removed creatine with the formation of a molecular equivalent of urea. Sarcosine was detected chromatographically but was not estimated.

Aerobic production of sarcosine by whole organisms. When suspensions of these organisms oxidize relatively low concentrations of creatine (6×10^{-3} M) the only detectable end products are urea, CO_2 and NH_3 (Nimmo-Smith & Appleyard, 1956). After incubation for 1 hr. with a higher substrate concentration (3×10^{-2} M) the presence of significant quantities of sarcosine was detected by paper chromatography; this disappeared on further incubation.

The oxidation of sarcosine to glycine and formaldehyde

The results in the preceding section suggest that the first step in the oxidation of creatine by suspensions of *Pseudomonas ovalis* Chester may be its hydrolysis to urea and sarcosine. If this is the case, and if it permeates the organisms, sarcosine itself should be rapidly oxidized. If sarcosine does not enter the organisms, the oxidation might be demonstrable with non-viable preparations provided that the enzyme systems are sufficiently stable.

Oxidation of sarcosine by whole organisms. Sarcosine was oxidized by suspensions of *Pseudomonas ovalis* under the usual conditions. As with creatine (Nimmo-Smith & Appleyard, 1956) a definite end-point was not obtained since, after the main reaction was over (and all sarcosine had been removed), the O_2 uptake continued indefinitely at slightly above the endogenous rate. Unlike creatine there was no sharp 'break' in the O_2 uptake curve (Fig. 4). Approximate values for O_2 and NH_3 at the end of the main reaction were 1.5 and 0.7 mole/mole sarcosine respectively; theoretical values for complete oxidation to CO_2 and NH_3 are 3.0 mole O_2 and 1.0 mole NH_3 .

The rate of oxidation of sarcosine (6×10^{-3} M) was only about a third of that with equimolar creatine. But as the concentration was raised the rate became equal to and finally exceeded the maximum rate obtained with creatine (Fig. 5).

Organisms harvested from a creatine-containing medium oxidized sarcosine about twice as rapidly as those from a similar medium containing glucose and ammonium salts in place of creatine (Fig. 4). With the latter organisms there was also a short lag before the full rate was established. It appears, therefore, that the sarcosine-oxidizing enzyme systems are at least partially adaptive.

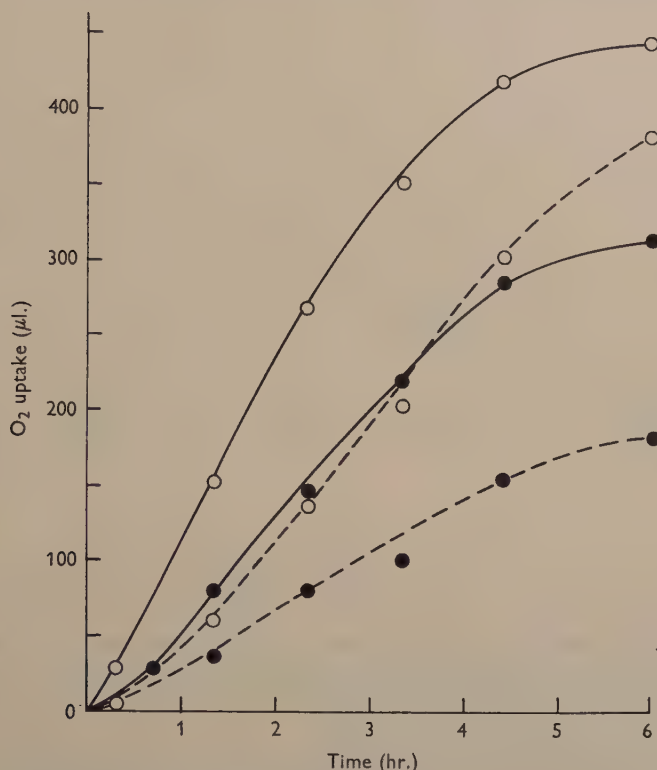


Fig. 4. Course of oxidation of sarcosine (○) and glycine (●) by *Pseudomonas ovalis* harvested from media containing creatine (—) or glucose and ammonium salts (---). Organisms (4.5 mg. dry wt.) incubated with 16.7 μ mole sarcosine or glycine in 0.067 M-phosphate buffer pH 7.8; total volume 2.5 ml.

The action of toluene-treated organisms. Of the three types of non-viable preparations from organisms used in this work only organisms treated simply with toluene retained the power to take up O₂ in the presence of creatine and sarcosine. Comparative Q_{O_2} values for whole and toluene-treated organisms showed 90% loss of activity in the case of creatine (Table 4). This may be partly explained by the loss of soluble creatinase in the supernatant fluid during the primary separation of the treated organisms from the toluene-water mixture. Centrifugation of the final preparation gave a supernatant fluid with creatinase activity, and repeated washing with water removed all such activity from the deposit, which retained, however, almost the full activity of the original preparation in oxidizing sarcosine. It is clear, therefore,

that with toluene-treated organisms the main pathway of creatine degradation is through sarcosine as intermediate.

The Q_{O_2} with sarcosine is 60% of that given by the original organisms. Glycine is not attacked, while formaldehyde is oxidized only very slowly

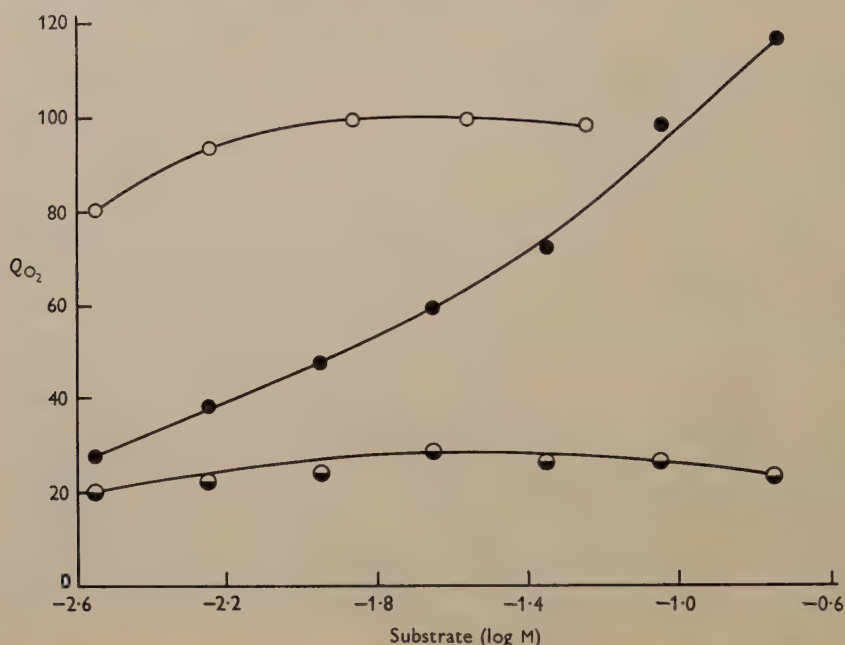


Fig. 5. Effect of substrate concentration on the rate of oxidation by cell suspensions of creatine (○), sarcosine (●) and glycine (◐). Organisms (4 mg. dry wt.) incubated in 0.067 M-phosphate buffer pH 7.8 plus substrates shown; total volume 3 ml.

Table 4. *Comparative rates of oxidation of various substrates by normal and toluene-treated Pseudomonas ovalis Chester*

Normal (6 mg. dry wt.) or toluene-treated (10 mg. dry wt.) organisms incubated in 0.067 M-phosphate buffer pH 7.8 with 16.7 μ mole substrate; total volume 3 ml.

Substrate	Normal organisms Q_{O_2} *	Toluene-treated organisms Q_{O_2} *
Creatine	100	9
Sarcosine	30	18
Glycine	20	0
Formaldehyde	25	2
None	25	1

* Figures corrected for the endogenous value given in the bottom line.

(Table 4). The rate of O_2 uptake with sarcosine falls to one-eighth of its original value by the time 0.6 mole O_2 /mole sarcosine has been used and sarcosine can no longer be detected in the reaction mixture. The slower rate of O_2 uptake continues indefinitely; it is probably due to the residual ability of the preparation

to oxidize formaldehyde which is one of the products of sarcosine oxidation; formaldehyde was detected by its colour reaction with chromotropic acid (see Methods).

The presence of glycine in the reaction products was established in three ways: (a) a ninhydrin-positive substance with an R_f in 'phenol' similar to that of glycine was detected on paper chromatograms; it separated from added serine and threonine, but not from glycine; (b) by fractionation on columns of Dowex 50; (c) by the growth response of *Leuconostoc mesenteroides* P60 (see Methods).

Table 5. *Products of the action of toluene-treated Pseudomonas ovalis on sarcosine*

Toluene-treated organisms (10 mg.) incubated in 0.08 M-phosphate buffer pH 7.8 with 16.7 μ mole sarcosine; total volume 2.5 ml.

Time (min.)	μ mole/ μ mole sarcosine initially present		
	O ₂ uptake	Formaldehyde formed	Glycine formed
30	0.18	0.31	0.35
100	0.48	0.82	0.82
190	0.58	0.70	0.98
Theoretical for reaction (3)	0.5	1.0	1.0

The results of quantitative experiments (Table 5) were in reasonable agreement with those expected for the reaction:



By the time (190 min.) that sarcosine could no longer be detected chromatographically the O₂ and formaldehyde values were respectively higher and lower than those theoretically required. This is probably accounted for by the slow oxidation of formaldehyde itself by the cell preparation; the figure for formaldehyde is actually higher at an earlier stage (100 min.). Some attempts to free the preparation from all ability to oxidize formaldehyde were not successful.

The enzyme catalysing reaction (3) will be termed sarcosine oxidase. It was not inhibited by 10⁻² M-sodium azide or by 90 % (v/v) carbon monoxide in the dark; cyanide (10⁻² M) inhibited completely, but was without effect at 10⁻³ M.

There was no evidence that sarcosine oxidase produced H₂O₂. The toluene-treated organisms have high catalase activity ($Q_{\text{O}_2} = 2500$) which is abolished by sodium azide (4×10^{-2} M). There was, however, no increased O₂ uptake with sarcosine in the presence of azide and no H₂O₂ could be detected.

Aerobic production of glycine by whole organisms. Glycine was detected chromatographically during the course of the action of suspensions of organisms on either sarcosine at ten times (6×10^{-2} M) the usual concentration or on either sarcosine or creatine (6×10^{-3} M) in the presence of 10⁻² M-sodium azide; this inhibitor causes a 50–60 % reduction in the rate of O₂ uptake with either creatine, sarcosine or glycine.

The oxidation of glycine and formaldehyde

Any hypothesis that the overall oxidation of creatine by whole organisms (reaction (1)) proceeds initially by the successive actions of creatinase and sarcosine oxidase (reactions (2) and (3)) demands that the organism should also contain enzymes catalysing the further oxidation of glycine and formaldehyde.

Glycine was oxidized by suspensions of *Pseudomonas ovalis*; but the Q_{O_2} was only about one-fifth of that with an equimolar concentration of creatine and did not increase appreciably when the glycine concentration was raised (Fig. 5). Quantitatively, the O_2 uptake and NH_3 formation fell short of the values required for complete oxidation of glycine (Table 6), but no other product was detected.

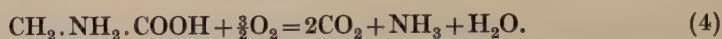


Table 6. *Oxidation of glycine and formaldehyde by suspensions of Pseudomonas ovalis*

Organisms (6 mg.) incubated in 0.067 M-phosphate buffer pH7 with substrate as indicated; total volume 3 ml.

Substrate	Amount (μ mole)	Q_{O_2}	μ mole/ μ mole substrate			
			O_2 uptake		NH_3 formed	
			Found	Theory*	Found	Theory*
Glycine	16.7	22	0.85	1.5	0.75	1.0
Formaldehyde	8.3†	25	0.6	1.0	—	—

* Theoretical values for complete oxidation according to reactions (4) and (5) (see text).

† Larger amounts were inhibitory.

Organisms harvested from the normal medium oxidized glycine about twice as rapidly as those from a medium in which creatine was replaced by glucose and ammonium salts (Fig. 4).

Formaldehyde was oxidized by suspensions of whole organisms ten times more rapidly than by the toluene-treated organisms (Table 4). An optimal Q_{O_2} was obtained with 3×10^{-3} M substrate; above this concentration the rate fell, probably due to a toxic action of formaldehyde on the oxidative mechanisms. The total O_2 uptake was only 60 % of that required for complete oxidation (Table 6).



DISCUSSION

It was shown in the preceding paper (Nimmo-Smith & Appleyard, 1956) that adapted *Pseudomonas ovalis* Chester oxidizes creatine with the overall production of urea, CO_2 and NH_3 . The complete oxidation of creatine (reaction (1)) would require values for O_2 , CO_2 and NH_3 of 3, 3 and 1 mole/mole creatine respectively; approximately 1.5, 1.5 and 0.7 mole were found. This discrepancy could be only partly accounted for by concurrent oxidative assimilation

(Clifton, 1946) of carbon and nitrogen into cell material; the highest values obtained in the presence of an uncoupling agent (sodium azide) were 2.0, 2.0 and 0.85 mole of O_2 , CO_2 and NH_3 respectively. The yield of urea was quantitative under all conditions. The fate of at least 25 % of the carbon and 5 % of the nitrogen of the creatine molecule is therefore unknown. Although it is possible that the extent of oxidative assimilation was greater than that demonstrated, and although no other final product was detected either by Nimmo-Smith & Appleyard (1956) or in the present work, it is clear that discussion of the pathway of creatine oxidation must take into account the possibility that there may be another undiscovered product of the overall reaction.

The present work provides good evidence that sarcosine is an intermediate in the breakdown of the bulk of the creatine and that glycine and formaldehyde may be later intermediates in a pathway for at least part of the creatine.

Sarcosine. The evidence in favour of sarcosine as a main intermediate is (a) organisms grown in media containing creatine (but not otherwise) contain an enzyme (creatinase) hydrolysing creatine to sarcosine and urea; it will be recalled that one mole of urea is invariably an end product of creatine breakdown. This enzyme can be obtained in a cell-free soluble form. (b) Sarcosine is oxidized with an uptake of O_2 (1.5 mole) and a production of NH_3 (0.7 mole) similar to that found with creatine itself; this would be expected since there is neither oxidation nor deamination in the conversion of creatine to sarcosine. (c) The rate of oxidation of sarcosine is doubled by growth of the organism on a medium containing creatine. The organism also grows well on a sarcosine medium (Nimmo-Smith & Appleyard, 1956). (d) Sarcosine can be detected during the course of creatine oxidation under certain conditions. (e) The organisms also contain an enzyme, sarcosine oxidase (present also in certain non-viable preparations), which catalyses the oxidation of sarcosine to glycine, and formaldehyde.

There are, however, certain difficulties. The apparent creatinase activity of whole organisms can be measured directly under anaerobic conditions: at a substrate concentration of $6 \times 10^{-3}M$ it is only one-fifth of that required to account for the aerobic removal of equimolar creatine. This does not appear to be due mainly to decreased permeability to creatine under anaerobic conditions since acetone-dried and crushed organisms are at the most 30 % more active than whole organisms (Fig. 2). The difference between the anaerobic and aerobic rates of creatine removal is abolished by raising the creatine concentration tenfold (Fig. 2). If the hydrolysis of creatine is also the first stage in creatine oxidation, it is necessary to assume that under aerobic conditions the organisms are able actively to concentrate creatine internally if the external concentration is too low.

There is a similar problem with regard to the oxidation of sarcosine. Only at relatively high substrate concentrations is the Q_{O_2} with sarcosine equal to or greater than that with equimolar creatine (Fig. 5). If sarcosine is an intermediate it is necessary to make the not unreasonable assumption that its formation within the organism produces an effective concentration in the locality of the enzyme concerned with its further metabolism.

Glycine and formaldehyde. The position of these substances as intermediates in the oxidation of creatine by intact organisms cannot be decided on the present evidence. In favour is the fact that they are the products of a sarcosine oxidase which survives in toluene-treated organisms and that both are oxidized by untreated organisms. Furthermore, glycine can be detected during the course of oxidation of creatine and sarcosine under certain conditions (p. 361). Glycine is also oxidized more rapidly by organisms harvested from a creatine medium (Fig. 4). Even allowing for the fact that only half the O_2 consumption for the complete oxidation of creatine (reaction (1)) would have to be attributed to glycine oxidation (reaction (4)), the Q_{O_2} obtained for whole organisms with glycine is only 40 % of that expected if it were an intermediate. The Q_{O_2} was not increased by raising the glycine concentration (Fig. 5), and it was not possible to test if the slow rate was due to limiting permeability of exogenous glycine, since none of the non-viable preparations tried oxidized this substrate.

The total O_2 uptakes with glycine and formaldehyde were less than those required for complete oxidation (Table 6), but no other products were detected. Oxidative assimilation with these substrates would be expected if they are intermediates; the phenomenon occurs with creatine itself and their oxidation requires five-sixths of the O_2 theoretically required for complete oxidation of creatine.

On the basis of the present results it is probable that at least part of the oxidation of creatine or sarcosine proceeds through glycine when the former are present at relatively high concentrations. The possible existence of another pathway (perhaps through some derivative of glycine*) which may predominate with lower substrate concentrations cannot be excluded.

Properties of sarcosine oxidase. This enzyme has previously been found in both animal tissues and bacteria. Mammalian liver preparations oxidize sarcosine (0.5 mole O_2 /mole) with the production of 1 mole of an amino-compound (presumed to be glycine) and an unknown amount of formaldehyde. The enzyme was insoluble, and it was concluded that electron transfer occurred through the cytochrome system (Handler, Bernheim & Klein, 1941; Bernheim & Bernheim, 1942). *Pseudomonas aeruginosa* also contains a sarcosine oxidase whose products are glycine and formaldehyde, though these were not obtained in quantitative yield (Kopper & Robin, 1950). This enzyme was completely inhibited by a concentration of cyanide ($10^{-3}M$) ten times less than that required with the *Pseudomonas ovalis* Chester enzyme. The present data are too scanty to permit a decision as to whether the cytochrome system is required for the bacterial enzyme.

This work was carried out during the tenure by one of us (G.A.) of a Medical Research Council Scholarship.

* N-formylglycine has been prepared but was not oxidized at an appreciable rate.

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Influence of Aliphatic Organic Acids and Metal Ions on Numbers of Local Lesions Produced by a Tobacco Necrosis Virus

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SUMMARY: Many aliphatic organic acids, when sprayed on the leaves of bean plants, decreased the numbers of local lesions produced following inoculation with a tobacco necrosis virus. Citric and succinic acids were effective only when applied before or during the period of virus establishment. The inhibitory effect of these acids could be annulled by certain metal nitrates.

The susceptibility of a species of host plant to a virus may vary greatly, as judged by the numbers of local lesions produced following inoculation with a standard preparation of virus. Age, nutrition, temperature, illumination and water supply are all known to affect susceptibility. Some of these factors alter the mechanical condition of the leaf, thus affecting the ease with which wounds necessary for virus entry are produced. Others are considered to alter 'host plant metabolism' but the chemical basis for such changes is unknown.

In an attempt to eliminate the daily cycle of variation in susceptibility to tobacco necrosis virus (Matthews, 1953) bean plants were grown under continuous illumination for several days before inoculation. Such plants were found to have a greatly decreased susceptibility to the virus compared with plants grown under natural illumination at about the same temperature. In most experiments no local lesions were produced at all, and no virus was detected in the inoculated leaves 9 days after inoculation. Preliminary analyses of such resistant leaves by chromatographic methods showed no major changes in sugar and amino acid content as compared with normally grown plants. However, there appeared to be a fairly marked increase in the amount of certain aliphatic organic acids. Subsequent tests described here showed that a variety of such acids, when sprayed on the leaves, decreased the number of lesions produced, an effect which was annulled by certain metal nitrates. The results suggest that metal ions may be important for the successful establishment of plant viruses and that one of the factors influencing plant susceptibility may be the balance between metal ions and organic acids in the leaf at the time of inoculation.

METHODS

The host plant, *Phaseolus vulgaris* var. Black Prince, and the tobacco necrosis virus were grown and inoculated as described previously (Matthews, 1953). Lesion counts were made 3-5 days after inoculation. Statistical analyses were made on transformed lesion numbers using the transformation $\log_{10} (x+1)$, where x = the number of local lesions per leaf.

RESULTS

The effect of treatment with organic acids on numbers of local lesions

Preliminary tests showed that when bean plants were sprayed with a solution of citric acid at *c.* 0.01N the numbers of local lesions produced by a standard inoculum were greatly decreased. Various organic acids (22 in all) were then tested by a standard procedure. Each acid at a concentration of 0.02N was sprayed on the two primary leaves of 12 plants. Four sprays were given—one on each of the 2 days before and after inoculation. Local lesions were counted after 5 days and the numbers compared with appropriate control

Table 1. *Effect of organic acids on numbers of local lesions produced by a tobacco necrosis virus in beans*

Acid	Decrease in number of lesions: <u>treated</u>	Significance of decrease (<i>t</i> test)	Leaf damage
	control	(%)	
Monocarboxylic			
Glyoxalic	0.60	5.0	Nil
Pyruvic	0.54	5.0	Slight
Glycollic	0.24	1.0	Nil
Lactic	1.80	5.0	Nil
α -Ketobutyric	0.75	Not significant	Nil
Dicarboxylic			
Oxalic	0.15	1.0	Marked
Malonic	0.32	1.0	Nil
Succinic	0.06	0.1	Very slight
Glutaric	0.14	1.0	Marked
Adipic	0.03	0.1	Marked
Oxaloacetic	0.15	0.1	Nil
α -Ketoglutaric	0.36	5.0	Nil
Malic	0.09	0.1	Nil
Fumaric	0.03	0.1	Moderate
Maleic	0.09	0.1	Nil
Mesaconic	0.08	0.1	Moderate
Itaconic	0.10	0.1	Heavy
Citraconic	0.03	0.1	Moderate
Tricarboxylic			
Tricarballic	0.14	1.0	Nil
Citric	0.21	1.0	Slight
Isocitric	0.13	0.1	Nil
Aconitic	0.06	0.1	Moderate

plants. The mean number of lesions/leaf on 192 control leaves was 46. The pH values of the solutions used for spraying ranged from 2.7 to 3.6. The results are summarized in Table 1. Some acids caused necrotic damage on the leaves. This was readily distinguished from the virus lesions. No correlation was apparent between the pH value of the solution sprayed, plant damage, and decrease in number of local lesions. The decrease in number of local lesions might have been due to some effect on the virus of the acid which remained on the surface of the leaf. This possibility was eliminated, at least for citric acid, by the following experiment. Bean plants were cut just above soil level and

the stems placed immediately in various concentrations of citric acid. After 24 hr. plants were inoculated with tobacco necrosis virus. Numbers of local lesions produced were (mean of 20 leaves): distilled water, 152; 0.00016N citric acid, 106; 0.00048N citric acid, 75; 0.0016N citric acid, 47. There was a progressive decrease in the numbers of local lesions.

Effect of treatment with metal nitrates on numbers of lesions

Metal ions are essential for infection by some bacterial viruses. Specific cations may be required, either for adsorption of phage on to sensitive bacteria (e.g. Puck, 1953) or for multiplication steps after phage attachment has taken place (Adams, 1949; Fildes, Kay & Joklik, 1951); no such evidence is available

Table 2. *Effect of succinic acid, magnesium nitrate and calcium nitrate on number of local lesions produced by tobacco necrosis virus in beans*

Spraying solution	No. of local lesions (mean of 16 leaves)	Significance of difference from	
		Control (%)	Sprayed with acid only (%)
Unsprayed control	19	—	0.1
Mg(NO ₃) ₂ , 0.001 M	27	1.0	0.1
Mg(NO ₃) ₂ , 0.01 M	36	0.1	0.1
Succinic acid, 0.01 N	8	0.1	—
Mg(NO ₃) ₂ , 0.001 M + succinic acid, 0.01 N	13	5.0	0.1
Mg(NO ₃) ₂ , 0.01 M + succinic acid, 0.01 N	24	None	0.1
Unsprayed control	84	—	0.1
Ca(NO ₃) ₂ , 0.001 M	77	None	0.1
Ca(NO ₃) ₂ , 0.01 M	70	None	0.1
Succinic acid, 0.01 N	20	0.1	—
Ca(NO ₃) ₂ , 0.001 M + succinic acid, 0.01 N	37	1.0	0.1
Ca(NO ₃) ₂ , 0.01 M + succinic acid, 0.01 N	49	1.0	0.1

for plant viruses. However, many organic acids are known to chelate metal ions. This suggested that if metal ions were essential for plant virus establishment organic acids might act, at least in part, by sequestering such ions. To test the effect of metal ions on the production of local lesions, plants were sprayed in the 4th and 2nd day before inoculation with an organic acid, and on the 3rd day and the day before inoculation with a metal nitrate. The results of one such experiment are summarized in Table 2. Both calcium and magnesium nitrates partially or completely annulled the effect of succinic acid. Magnesium nitrate alone at 0.01M increased the number of local lesions about twofold. Various other nitrates were tested in experiments like that recorded in Table 2. Potassium and ammonium nitrates had no effects. In some experiments sodium nitrate significantly reduced the effect of succinic and citric acids. Caesium, barium and aluminium nitrates applied alone significantly increased the number of local lesions and also reduced the inhibitory effect of succinic acid. Zinc nitrate alone caused a striking reduction in number of lesions, as has been found previously for tobacco mosaic virus (Weintraube, Gilpatrick & Willison, 1952).

The extent to which numbers of local lesions were decreased by citric and succinic acids varied in different experiments, as did the annulling effect of metal ions. Until the factors affecting this variation are understood it is not possible to give firm comparative figures for the effects of different metals.

Age of plant does not appear to affect the activity of succinic acid. Plants in four age groups, ranging from those in which the leaves were not fully expanded to plants approaching senescence, gave the following figures for the ratio of number of local lesions on succinic acid treated plants: control, namely 0.15, 0.13, 0.14, 0.13 in order of increasing age of plant.

The effect of time of treatment in relation to time of inoculation

Preliminary experiments showed that a single treatment with an organic acid was effective when applied 1 or 2 days before inoculation, but ineffective when applied 1 day after inoculation. The effect of single treatments at times close to the time of inoculation was then examined. Since spraying with water alone just before inoculation caused a marked decrease in numbers of local lesions, a control set of plants was sprayed with water for each time tested. The results of one experiment are summarized in Table 3.

Table 3. *Effect of 0.02N succinic acid applied at various times before and after inoculation*

Time of spraying in relation to time of inoculation	Ratio of number of local lesions: acid sprayed water sprayed
15 min. before	0.14
Immediately before	0.28
Immediately after	0.50
15 min. after	0.56
30 min. after	0.56
45 min. after	0.67
60 min. after	0.55
1.5 hr. after	0.71
2 hr. after	0.82

A treatment immediately before inoculation decreased the number of lesions more than a treatment immediately after inoculation. In various experiments treatments with succinic or citric acids after about 2-4 hr. were ineffective.

DISCUSSION

The fact that the decrease in numbers of lesions by organic acids was annulled by metal ions does not necessarily imply a close connexion between action of these two types of compound. However, some organic acids are known to sequester metal ions through the formation of chelate compounds. The simplest explanation for the effects described here is to assume that the tobacco necrosis virus requires certain metal ions for successful establishment and that the organic acids inhibit this process by decreasing the concentration of

available metal ion. However, in preliminary tests the well-known chelating agent, ethylenediamine tetra-acetic acid caused no greater decrease in lesion number than did citric and succinic acids.

With bacterial viruses it is possible to control the concentration of compounds in the medium in which virus establishment occurs. No such control is possible with plant leaves. The variation in the magnitude of the effects we have observed may well be due to natural variation in the state of the plant with respect to metal ions and organic acids or other chelating compounds at the time of inoculation. Some of the acids on entering the leaf are probably rapidly metabolized. In addition, there is no reason to suppose that all the acids listed in Table 2 exert their inhibitory effect in the way suggested above. For these reasons no correlation can be expected between data such as are given in Table 1 and the chelate stability constants for the various acids with calcium and magnesium (Martell & Calvin, 1952). It may be of interest from the point of view of virus control that a class of compound which must be universally present in susceptible leaves can in increased concentration inhibit virus establishment.

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The Wavelengths of Helical Bacterial Flagella

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SUMMARY: During normal movement most motile bacteria carry a straight tail, which, when the movement slows, stiffens into helical structures commonly called flagella. The helices of many kinds of bacteria were photographed with a sunlight dark-ground microscope, and their wavelengths measured. Mean values and standard deviations were calculated for each strain and then for the species. 'Biplicity' (two wavelengths per bacterium, one twice the other) was observed frequently. Each strain appears to have its own constant wavelengths. The wavelength differs in different kinds of bacteria from 0.60 to 5.058 μ ., the distribution over the various species not revealing a distinct pattern nor any obvious correlation with other characteristics. The wavelength is affected by temperature, pH value, and colloid content of medium. These features, and the effects of drying, make stained preparations useless for measuring.

In sunlight dark-ground microscopy most motile bacteria, except spirilla (Pijper, 1949*a*, 1955*c*; Pijper, Crocker, van der Walt & Savage, 1953) and vibrios (Pijper & Nunn, 1949) during normal forward movement show a fuzzy-looking straight tail, as in Pl. 1, fig. 1 (Pijper, 1946, 1949*b*). As cultures age, and more rapidly in slide-coverslip preparations, these tails stiffen into clear-cut rods, or, more frequently into clear-cut helices (Pijper & Abraham, 1954; Pijper, 1955*a*; Pijper, Crocker & Savage, 1955). Pl. 1, figs. 2-4, show the shortest and the longest wavelengths so far found. 'Wavelength', also called 'period' or 'pitch', is distance from crest to crest. Helices in one culture may be of two different wavelengths, one twice the other (Pijper & Abraham, 1954; Pijper *et al.* 1955). For this 'doubling' phenomenon the term 'biplicity' was suggested (Pijper, 1955*a*). Helices often 'link up' lengthwise and 'pack' crosswise, thus forming 'giant-flagella' (Pl. 1, fig. 5). Slight degrees of packing as in Pl. 1, figs. 3 and 6, do not affect wavelengths.

Reichert (1909), in dark-ground microscopy of colloid-thickened helices, estimated the pitch of these 'screw-like flagella' of *Salmonella typhi* as 2.5 μ ., those of *Proteus vulgaris* as 2 μ ., and those of sarcinas as usually 3 μ ., occasionally 2 μ . or even 1.8 μ . Weibull (1949) precipitated shaken-off flagella of one strain each of *P. vulgaris* and *Bacillus subtilis* with ammonium sulphate and described the wet helices (really giant-flagella) as having a period of 2 μ . in *Proteus vulgaris* and 2.5 μ . in *Bacillus subtilis*, later confirming the value of 2 μ . on ten strains of *Proteus vulgaris* (Weibull, 1950); he concluded that a definite spiral period is a characteristic feature of bacterial flagella. Leifson (1951), working with bacteria dried and stained on slides, got the impression that perhaps the degree of curvature of flagella might be more important than total length. Recently Leifson, Carhart & Fulton (1955), again using dried flagella-stained bacteria, measured one single wavelength of 10 'flagella' of each of one

75 strains of *Proteus* and found large differences between otherwise identical strains, in our opinion caused by the drying process. They did not comment on the 'biplicity' discernible even in their dried and distorted preparations. Their suggestion that the proportion of 'curly' and 'normal' flagella (their names for flagella of obviously single and double wavelengths) is affected by pH value is not borne out by their figures. Peluffo (1953), whose technique gave us numbers of giant-flagella just as easily visible to us in ordinary microscopy as in phase contrast, measured unknown numbers of these structures by phase-contrast microscopy and found values for *Pseudomonadaceae*, salmonellas, and *P. vulgaris* of about the same order as ours.

We have watched the genesis of the helical structures in a number of different bacteria, and, under standardized conditions, which we found essential, examined them for wavelength and presence of biplicity.

METHODS

Organisms. We classified our bacteria after *Bergey's Manual* (1948), applying the diagnostic methods given there. The salmonellas were also classified serologically.

Photomicrography and measurements. When a sufficient number of tails had stiffened into helices as watched under the microscope, they were photographed with the sunlight dark-ground technique described before (Pijper, 1946). Electric lamps were inadequate, at any rate for the finer helices. With a Zeiss apochromat $\times 60$ and a Contax camera the magnification on the film was $\times 300$. For each strain of bacteria at least one full reel of thirty-six pictures was used. As a rule each reel made over 100 photomicrographs of helical structures available for measuring, and as most of them consisted of more than one wavelength, our calculations for each strain usually were based on several hundreds of wave lengths. The exceptions are specially mentioned. For measuring, the negatives were projected downwards in a vertical photographic enlarger with a Zeiss Sonnar 1:2 at a total magnification of $\times 3000$, on to sheets of white paper. The clear-cut helices were traced with a pencil and then measured with vernier calipers allowing 0.1 mm. to be read. Instances of the very regular frequency curves resulting from this technique are shown in Fig. 1. Mean wavelength and standard deviation (s.d.) were calculated from the millimetre values at the magnification of $\times 3000$. To facilitate understanding and comparison we give them here divided by 3 so as to express them in microns (μ). However, we have kept all the decimals so that our original figures can be obtained by multiplying by 3. We do not ascribe accuracy to our figures beyond the second decimal.

RESULTS

Effect of temperature

Appliances for heating and cooling the microscope stage described elsewhere (Pijper, 1955*b*) were used for measuring helices at wide ranges of temperature. Table 1 shows that wavelengths increased with rises in temperature. The

naturally shorter wavelengths were less affected by heat than the longer ones. The observations were repeated with similar results. As a rule a fresh preparation was made for each temperature observation and each preparation was left on the stage for at least an hour before photomicrographs were made. We once kept a slide of *Bacillus megaterium* 7581 first in a refrigerator at 6° overnight, which shortened the wavelengths. It was then brought to 47° on the microscope stage and showed a wavelength of 3.564 μ . (s.d. 0.153) (see Table 1). After this it was cooled on the microscope stage to 3° for 3 hr. and

Table 1. *Effect of temperature of microscope stage on helical wavelengths*

Wavelengths are given in μ ., followed by the standard deviation in brackets. Values recorded are based on at least 100 measurements.

Name of bacterium	Temperatures		
	3°	16-19°	47°
<i>Salmonella typhi</i> 901	2.289 (0.059)	2.294 (0.065)	2.441 (0.062)
<i>S. schottmuelleri</i> 3176	2.234 (0.053)	2.267 (0.075)	2.381 (0.052)
<i>Bacillus megaterium</i> 7581	3.389 (0.111)	3.432 (0.130)	3.564 (0.153)
<i>Serratia marcescens</i> 2302	0.941 (0.037)	0.965 (0.037)	0.950 (0.032)

photographed again, it then showed a wavelength of 3.392 μ . (s.d. 0.131), corresponding very closely to the value for a fresh preparation at that same low temperature given in Table 1 as 3.389 μ . (s.d. 0.111). A preparation of *Salmonella schottmuelleri* 3176 at 15° had a wavelength of 2.245 μ . (s.d. 0.044), and this when brought to 25° became 2.299 μ . (s.d. 0.048). Because of these, and other similar findings, we made our further measurements within the temperature range of 16-19°. During the colder months the microscope room stayed between 16 and 19°, during the hot weather ice-water flowing through a small tank clamped to the microscope stage maintained this same range of temperature (Pijper, 1955b).

Effect of pH value

By adding hydrochloric acid or sodium hydroxide to cultures before measuring, the pH values were adjusted as shown in Table 2. Readings of pH values were made by indicators and checked with a Beckman pH meter. The actual values between slide and coverslip probably deviated somewhat from the intended ones; the effect of the platinum loop, the mica slide (mica was used to get a clearer background), the glass coverslip and further growth during examination was not evaluated. The main finding was that wavelengths were shortest at pH 7.0, increased alkalinity and acidity increasing the wavelengths (Table 2). As with the effect of heat, the naturally shorter wavelengths were least affected. The results were confirmed by measuring wavelengths on *Salmonella typhi* 901 suspended in phosphate buffer solutions of pH 6.05 and

10.0 (determined with the Beckman pH meter), and finding the wavelengths 2.366 μ . (s.d. 0.064) and 2.353 μ . (s.d. 0.061) respectively, both values being higher than the values at pH 7.0. In a similar experiment at 20–25° we found a wavelength of 2.315 μ . (s.d. 0.059) at pH 6.0, and a wavelength of 2.350 μ . (s.d. 0.058) at pH 8.0, both values again being higher than the value at pH 7.0. Because of these findings our further measurements were undertaken in media adjusted to pH 7.0.

Table 2. *Effect of pH value on helical wavelengths*

Values for wavelengths in μ . (based on at least 100 measurements); standard deviation in brackets.

Organism	pH values							
	4.0	4.5	5.0	6.0	7.0	8.0	9.0	10.0
<i>Salmonella typhi</i> 901	—	—	2.336 (0.084)	2.312 (0.047)	2.303 (0.051)	2.367 (0.054)	2.362 (0.050)	2.366 (0.065)
<i>S. schottmuelleri</i> 3176	2.305 (0.059)	—	2.292 (0.044)	—	2.267 (0.051)	—	2.294 (0.050)	2.284 (0.041)
<i>Sarcina ureae</i>	—	—	—	—	3.055 (0.100)	—	3.218 (0.074)	3.323 (0.063)
<i>Proteus mirabilis</i> 3177	—	1.892 (0.092)	—	1.874 (0.055)	1.872 (0.072)	1.865 (0.059)	1.861 (0.069)	—

Effect of colloid substances

Colloid substances such as gelatin, gums, agar and methylcellulose when present in bacterial suspensions precipitate on the bacteria and thus coat bodies, tails and helices, making them more readily visible in dark-ground microscopy (Pijper, 1947). We found that the wavelengths of helices, whilst not markedly affected by methylcellulose, were affected by other colloids. *Salmonella typhi* 901, grown and examined in plain broth, showed a wavelength of 2.289 μ . (s.d. 0.074) and in 1 % (w/v) methylcellulose broth of 2.294 μ . (s.d. 0.065). In 10 % (w/v) gum-arabic broth the wavelength was 2.423 μ . (s.d. 0.089), and when grown on nutrient agar and suspended in the water of condensation it was 2.316 μ . (s.d. 0.070). *S. typhi* 2 in plain broth had a wavelength of 2.289 μ . (s.d. 0.053) and in 1 % methylcellulose broth, 2.274 μ . (s.d. 0.063). *Bacillus subtilis* 7197 in 1 % methylcellulose broth showed a wavelength of 2.157 μ . (s.d. 0.096) and on agar it was 2.277 μ . (s.d. 0.042). Because of these findings we subsequently avoided other colloids and used methylcellulose. In a few cases, where no helices appeared in methylcellulose broth, agar was used, and this is specially mentioned.

Effect of going rough

Several of our strains were old and going rough. Prof. Winkler gave us a smooth form of *Salmonella dublin* and its rough variant. The smooth form was more motile and had longer tails and helices than the rough variant. A total of 179 measurements on the smooth form gave 2.355 μ . (s.d. 0.066) and 115 measurements on the rough variant gave 2.354 μ . (s.d. 0.096). Obviously going

rough did not affect wavelength. Because of the better development of tails and helices in smooth forms, wherever possible, we picked the smoothest colonies of our strains. Arena & Schwartz (1937), in stained preparations, saw fewer flagella in rough *S. typhi* than in smooth, but the difference in shape which they described and depicted (more 'wider undulations' in the smooth and more 'narrower undulations' in the rough form) evidently were examples of biplicity and not smooth-rough variation, as they thought.

Effect of drying

Flagella staining is preceded by drying which distorts, dislocates and redistributes the helical structures on the slide (Pijper, 1946). The flagella of Pl. 1, fig. 7, were stained by the method of Leifson (1951), and show marked dissimilarity. In such pictures one can pick out some rather regular and matching shapes, and for measuring purposes the temptation to do so is great, but selection is not fair sampling. In our dark-ground photomicrographs all visible helices could be used without selection, as illustrated by Pl. 2, fig. 8. Fig. 1 shows frequency curves of helical wavelengths for four strains of bacteria. A curve was drawn for each one from dark-ground and from stained picture measurements. The former are symmetrical with very little scatter, the latter are so irregular that mathematical analysis does not seem worth while, and the mean values of the latter would obviously differ greatly from those of the dark-ground technique. Apart from changes caused by drying the effects of changes in pH value and in temperature during the staining process must be considerable. Because of all this little value can be attached to measurements of stained flagella.

Helical wavelengths of various bacteria

We give the mean helical wavelength of each strain, and by formal calculation taking into account the number of measurements on each strain, the mean wavelength of the species. There follows a list of the results; the mean wavelengths are given in μ , followed by the standard deviation (s.d.) in brackets.

Salmonella typhi (Pl. 2, fig. 8). Mean wavelengths of twelve strains: 2.274 (0.063), 2.276 (0.051), 2.282 (0.060), 2.288 (0.067), 2.289 (0.057), 2.293 (0.061), 2.294 (0.065), 2.295 (0.047), 2.299 (0.059), 2.301 (0.068), 2.304 (0.056), 2.315 (0.050). Overall mean: 2.293 (0.061). Biplicity: in broth culture *S. typhi* 901 once showed 34 helices of mean wavelength 1.106 (0.039) and in methylcellulose-broth 4 helices averaging 1.2 μ . (Pl. 2, fig. 9.)

Salmonella paratyphi. Mean wavelengths of six strains: 2.308 (0.083), 2.340 (0.055), 2.342 (0.054), 2.367 (0.077), 2.371 (0.064), 2.421 (0.058). Overall mean: 2.340 (0.078).

Salmonella schottmuelleri. Mean wavelengths of seven strains: 2.252 (0.068), 2.267 (0.075), 2.282 (0.049), 2.298 (0.046), 2.301 (0.055), 2.302 (0.047), 2.335 (0.061). Overall mean: 2.289 (0.064). One strain, 3176, showed abundant biplicity: in one preparation 211 helices of 2.267 (0.075) and 108 helices of 1.131 (0.059). (Pl. 2, fig. 10.)

Salmonella hirschfeldii. Mean wavelengths of four strains: 2.352 (0.084), 2.385 (0.071), 2.409 (0.070), 2.455 (0.063). Overall mean: 2.400 (0.084).

Salmonella typhimurium. Mean wavelengths of ten strains: 2.267 (0.073), 2.287 (0.056), 2.298 (0.054), 2.335 (0.103), 2.344 (0.063), 2.345 (0.070), 2.349

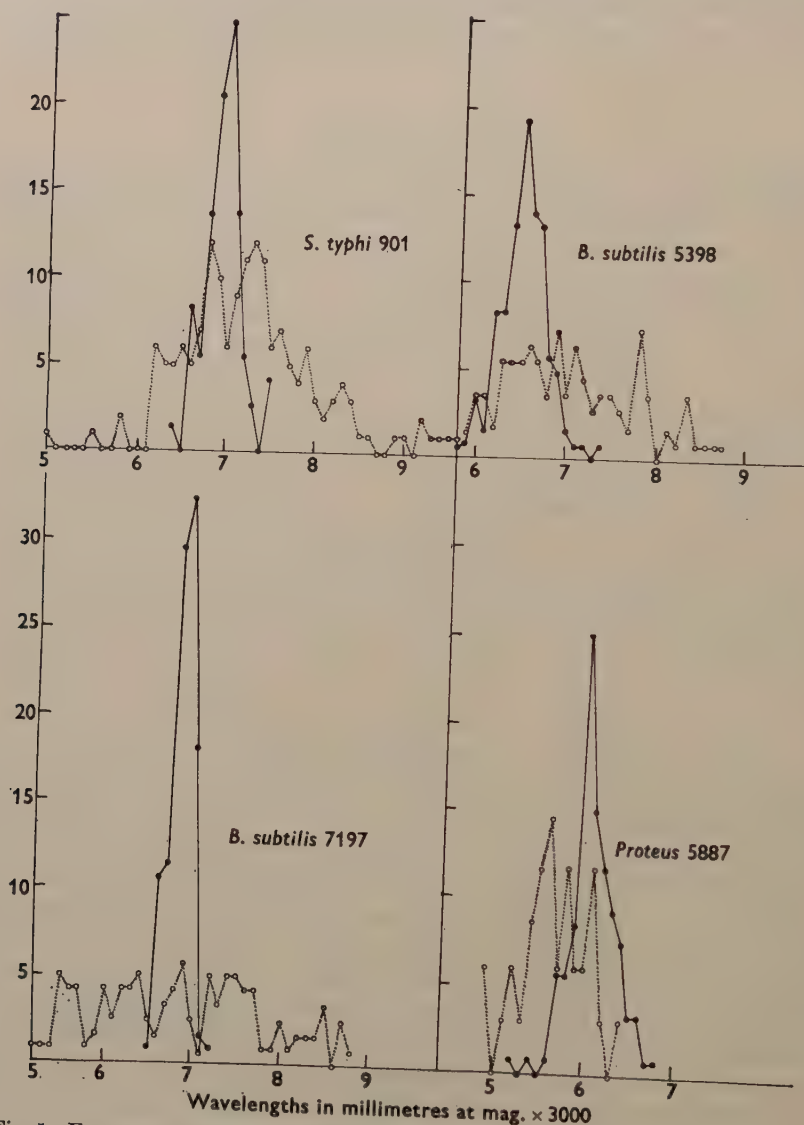


Fig. 1. Frequency curves of wavelengths of helical bacterial flagella. ●—●, dark ground; ○—○, stained.

(0.084), 2.384 (0.081), 2.407 (0.068), 2.452 (0.081). Overall mean: 2.350 (0.091). Biplicity: few wavelengths averaging 1.2μ . in one strain.

Salmonella enteritidis. Mean wavelengths of three strains: 2.311 (0.109), 2.328 (0.070), 2.370 (0.064). Overall mean: 2.335 (0.088).

Salmonella dublin. Mean wavelengths of two local strains from Prof. Henning: 2.279 (0.087) and 2.280 (0.087). The German strain mentioned above gave 2.355 (0.066). Fermentatively the three strains were identical, including action on rhamnose (Kauffmann, 1951).

Proteus mirabilis. Mean wavelengths of nine strains: 1.919 (0.066), 1.928 (0.068), 1.929 (0.074), 1.933 (0.054), 1.945 (0.071), 1.974 (0.060), 2.007 (0.064), 2.031 (0.076), 2.031 (0.086). Overall mean: 1.962 (0.081).

Proteus vulgaris. Mean wavelengths of two strains: 2.049 (0.093), 2.143 (0.063). Overall mean: 2.098 (0.092). Biplicity: a few helices of average wavelength of about 1μ . (Pl. 2, fig. 11.)

Bacillus megaterium. Mean wavelengths of four strains: 3.298 (0.205), 3.362 (0.101), 3.432 (0.130), 3.451 (0.145). Overall mean: 3.389 (0.166). No biplicity but one strain showed a number of shorter wavelengths averaging 1.1μ . (Pl. 2, fig. 12, long wavelength.)

Bacillus cereus. Mean wavelengths of eight strains: 2.169 (0.088), 2.204 (0.081), 2.255 (0.075), 2.329 (0.066), 2.392 (0.060), 2.479 (0.095), 2.516 (0.104), 2.567 (0.066). Overall mean: 2.356 (0.164). Biplicity: the strain of 2.169 showed some wavelengths of about 1.2μ . (Pl. 2, fig. 13), and the strains of 2.516 and 2.567 had a number of wavelengths averaging 1.3μ .

Bacillus subtilis. Mean wavelengths of eight strains: 2.138 (0.099), 2.144 (0.104), 2.154 (0.066), 2.157 (0.096), 2.158 (0.082), 2.166 (0.100), 2.229 (0.060), 2.295 (0.080). Overall mean: 2.186 (0.103). Biplicity: four strains had some wavelengths of about 1.1μ .

Bacillus pumilus. Mean wavelengths of six strains: 2.333 (0.122), 2.335 (0.096), 2.348 (0.112), 2.349 (0.123), 2.374 (0.094), 2.378 (0.123). Overall mean: 2.353 (0.112). Biplicity: one strain had some wavelengths of about 1.2μ .

Pseudomonas aeruginosa. Mean wavelengths of three strains: 1.477 (0.048), 1.502 (0.076), 1.552 (0.086). Overall mean: 1.530 (0.086). Helices were scanty and the number measured in two strains less than 100.

Pseudomonas diminuta. Leifson & Hugh (1954) made this a new species from the very short wavelength of its stained flagella (0.62μ). We found scanty helices and measured 17 with an average wavelength of 0.60μ . (Short wavelength helices are always less affected by outward circumstances) (Pl. 1, figs. 2, 3).

Pseudomonas of undetermined nature, from retting tank. Mean wavelength of 121 measurements: 1.342 (0.075).

Azotobacter agile, from local soil. Mean wavelength of 86 measurements: 2.060 (0.074).

Azotobacter insignis. Mean wavelength of 164 measurements on the strain from Derx (1951) 5.058 (0.157) (Pl. 1, fig. 4).

Aerobacter aerogenes. Mean wavelengths of five strains: 2.247 (0.067), 2.285 (0.086), 2.352 (0.085), 2.365 (0.110), 2.419 (0.107). Overall mean: 2.336 (0.114).

Escherichia coli. Mean wavelength of four strains: 2.278 (0.108), 2.315 (0.101), 2.379 (0.103), 2.497 (0.095). Overall mean: 2.366 (0.121). Biplicity: two strains had some wavelengths of about 1.15μ .

Serratia marcescens. Mean wavelengths of three strains: 0.965 (0.037), 2.346 (0.080), 2.591 (0.108). The first one produced a variant with a mean wavelength of 0.979 (0.050). The values are too divergent for an overall mean.

Serratia indica and *S. kilensis*. One strain each with practically identical wavelengths: 2.651 (0.117) and 2.649 (0.089).

Chromobacterium ianthinum. One strain, producing helices on agar only. Mean wavelength: 1.115 (0.035).

Caryophanon latum. One strain, identified by Pringsheim & Robinow (1947), showed helices on acetate yeast extract medium only. Measurements once done on 84 helices gave a mean of 1.821 (0.081) and on another occasion on 135 helices 1.831 (0.054). Biplicity: some wavelengths averaging 0.9 μ .

Nocardiaturbata. Strain received from Dr Dagny Erikson and described by her (1954) produced a number of motile organisms on agar, sometimes branching, often detached. Mean wavelength of 52 helices (Pl. 2, fig. 14): 2.500 (0.125).

Sarcina ureae. The mean value previously given (Pijper & Abraham, 1954) as 3.193 (0.0048) was determined in an alkaline medium without regard to temperature; at pH 7.0 and temperatures between 16 and 19° it was 3.055 (0.100). Biplicity again was abundant.

DISCUSSION

We can see no 'pattern' in the distribution of the various wavelengths over the different species, nor is there a correlation between wavelength and other attributes. Three wavelengths were observed in *Serratia marcescens* and at least two in *Pseudomonas* spp. *Bacillus megaterium* and *B. cereus* both have large diameters, but their wavelengths are very different. *Caryophanon latum* with its large body has a relatively short wavelength. The salmonellas tended to have wavelengths of about 2.3 μ , but there seem to be distinct differences between the members of this group, and not all strains of *Salmonella dublin* had the same wavelength. Biplicity was a varying phenomenon, frequent in some strains and rare or absent in closely allied ones.

Helical wavelengths, however typical in some strains (*Pseudomonas aeruginosa* and *P. diminuta*, salmonellas in general, *Bacillus megaterium*, *Sarcina ureae*, *Azotobacter insignis*), will be of limited use for classification. There is too much overlapping, the differences are not very great, and there always is a possible latent biplicity. As temperature, pH value and colloid substances affect wavelengths, accurate measurement is an involved process.

We have witnessed the transition from straight tail to helix in nearly all our bacteria. Exceptions were the pseudomonads, the azotobacters and the nocardia, where we saw no straight tails; perhaps these were too thin. When preparations of these bacteria were left on the stage for some hours, until motility had ceased, helices became visible, though usually in small numbers. Finally, we emphasize that in spirilla there is no transition from straight tail to helix; flagella with a typical twist, which is not helical, are present all the time in spirilla (Pijper, 1955c).

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EXPLANATION OF PLATES

All magnifications are $\times 2000$, except Pl. 2, fig. 8, which is $\times 1000$.

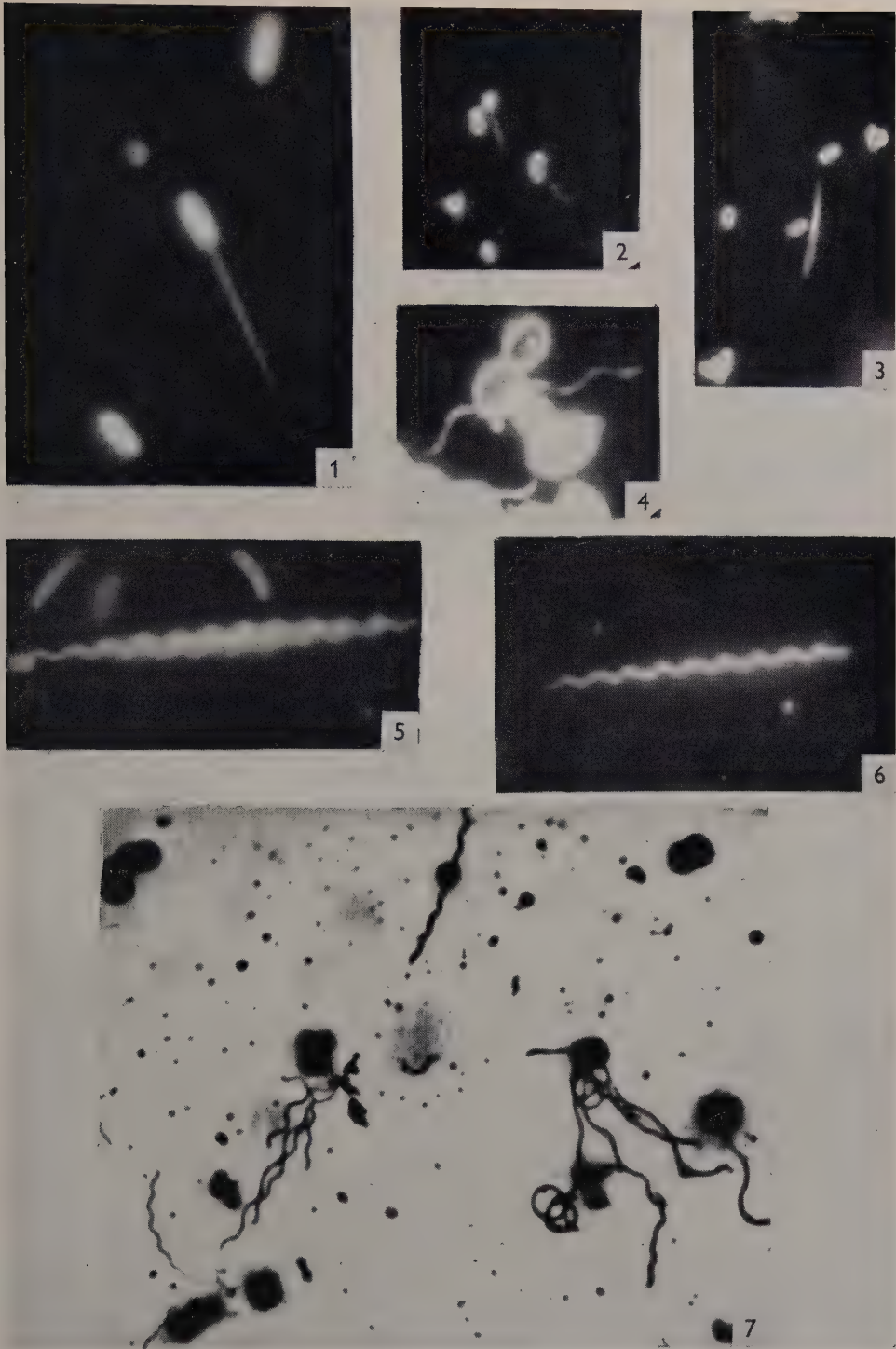
PLATE 1

- Fig. 1. *Salmonella schottmuelleri* 3176.
Figs. 2, 3. *Pseudomonas diminuta*.
Fig. 4. *Azotobacter insigne*.
Fig. 5. Giant-flagellum of *Salmonella schottmuelleri* 3176.
Fig. 6. Giant-flagellum of *Bacillus cereus* 7464.
Fig. 7. *Sarcina ureae* stained with Leifson's method for flagella.

PLATE 2

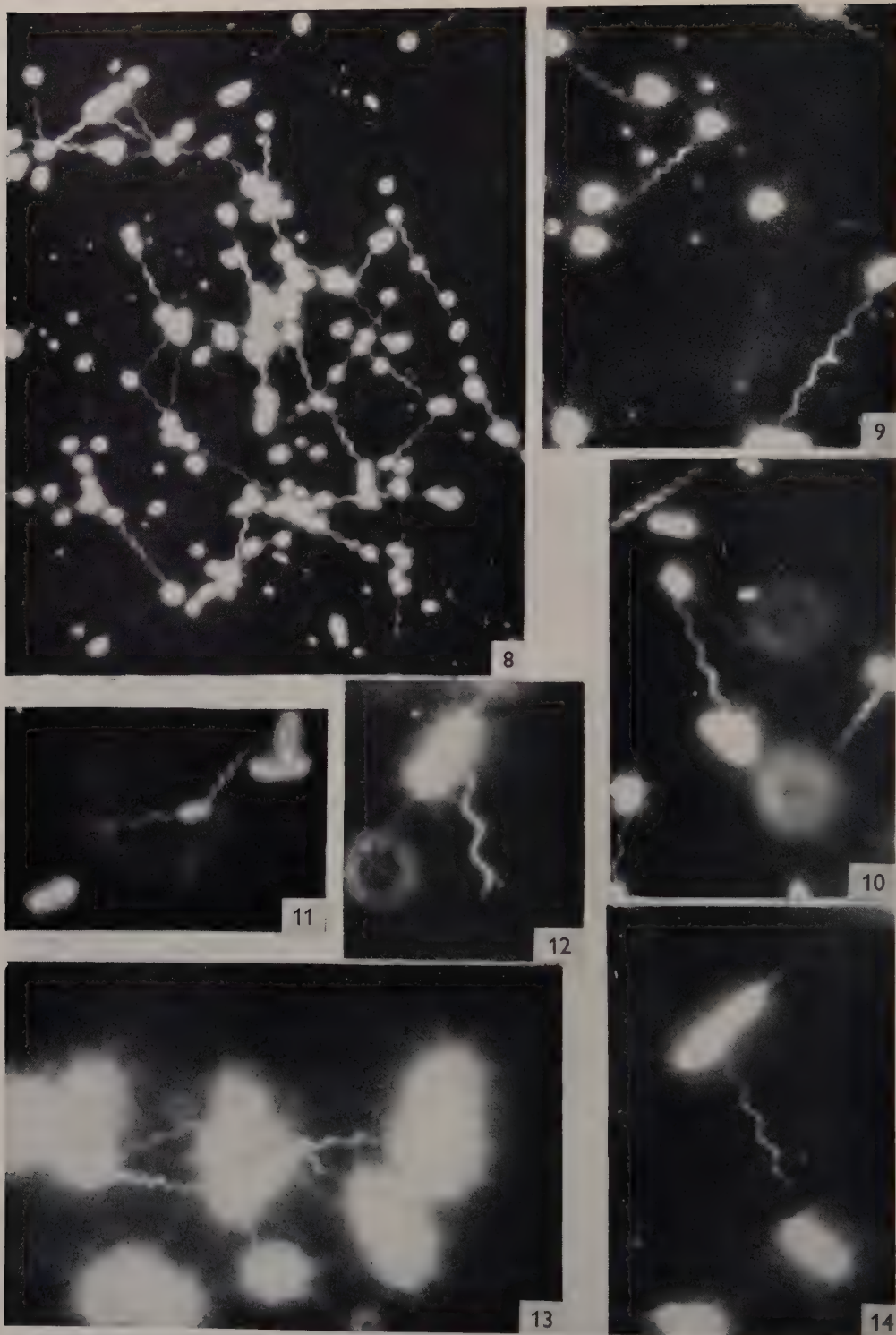
- Fig. 8. *Salmonella typhi* 901.
Fig. 9. *S. typhi* 901, showing biplicity.
Fig. 10. *S. schottmuelleri* 3176, showing biplicity.
Fig. 11. *Proteus vulgaris* 4175, showing biplicity.
Fig. 12. *Bacillus megaterium* 7581.
Fig. 13. *B. cereus* 7464, showing biplicity.
Fig. 14. *Nocardia turbata*.

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A. PIJPER, M. L. NESER & G. ABRAHAM—WAVELENGTHS OF HELICAL BACTERIAL FLAGELLA.
PLATE 1

(Facing p. 380)



A. PIJPER, M. L. NESER & G. ABRAHAM—WAVELENGTHS OF HELICAL BACTERIAL FLAGELLA.
PLATE 2

SKINNER, F. A. (1956). *J. gen. Microbiol.* **14**, 381-392

Inhibition of the Growth of Fungi by *Streptomyces* spp. in Relation to Nutrient Conditions

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SUMMARY: Several species of soil actinomycetes arrested the growth of fungi by antibiotic secretions on agar media containing 10 g. glucose/l. On media of lower glucose concentration, the fungi continued to grow in the presence of the actinomycetes, but there was evidence that traces of antibiotic substances were still being formed. In sand moistened with liquid medium containing glucose, *S. albidoflavus* limited early growth of *Fusarium culmorum* by antibiotic action and also attacked preformed fungus mycelium directly. The effectiveness of these antagonistic mechanisms was decreased when the glucose concentration was lowered. The fungus and the actinomycete grew together on a variety of natural organic materials, but only when dried grass was used did the actinomycete arrest growth of the fungus at a distance.

Actinomycetes are numerous in soils (Waksman, 1950), and a high proportion of strains isolated can antagonize bacteria and fungi in culture (Waksman, Horning, Welsch & Woodruff, 1942; Schatz & Hazen, 1948). There is no doubt that most examples of antagonism encountered in mixed cultures are due to the production of inhibitory substances by the actinomycetes. These antagonistic effects are often so striking that it is tempting to consider such substances as being factors of considerable importance in influencing the balance of natural soil populations. Nevertheless, though many species of soil actinomycetes can produce their antibiotics in artificial culture when supplied with nutrients of the right kinds in adequate amounts, it is by no means certain that they can form the antibiotics in sufficient quantity to antagonize other micro-organisms in soil where the quality and amounts of nutrients may not permit rapid growth (Robison, 1945; Waksman, 1945). A study has accordingly been made of the ability of some soil actinomycetes to suppress the growth of fungi, particularly by the production of antifungal inhibitors, when growing under conditions unfavourable to luxuriant development.

METHODS

Organisms

Five test fungi (*Fusarium culmorum*, *Rhizoctonia goodyerae-repentis*, *Fusarium* sp., *Stemphylium* sp., and an unidentified sterile mycelial form) were used in preliminary tests for selecting soil actinomycetes which displayed antibiotic activity. Five species of *Streptomyces*, each strongly inhibitory to the growth of all the five test fungi on several different agar media, were isolated from local soils. In most of the experiments one of these actinomycetes, identified

as *Streptomyces albidoflavus* (Rossi Doria) comb.nov. (Skinner, 1951) was used with *Fusarium culmorum*, a strain of which was obtained from Dr W. J. Dowson of the Botany School, Cambridge.

Media

Artificial soil solution media. Solid and liquid media were prepared from an 'artificial soil solution' similar to that described by Erikson (1947). This solution (hereafter 'AS solution') consisted of (g./l. distilled water): CaSO_4 , 0.8; $\text{Ca}(\text{NO}_3)_2$, 0.33; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.7; K_2SO_4 , 0.025; K_2HPO_4 , 0.005; NaHCO_3 , 0.2; FeCl_3 , trace. This solution plus specified amounts of glucose was used to moisten sand cultures; it was also used as a solid medium by the addition of 1.5% (w/v) water-washed agar. These media were sterilized by steaming for 20 min. on 3 successive days. The pH value was adjusted to 7.0–7.2.

Plain buffered agar medium (pH 7.0) was made by dissolving 15 g. agar in 500 ml. buffer solution and sufficient distilled water to make 1 l. (This volume of buffer was made by mixing 195 ml. of M/15 KH_2PO_4 and 305 ml. of M/15 Na_2HPO_4 .) The medium was autoclaved for 15 min. at 15 lb./sq. in.

Glucose asparagine solution. Glucose, 10 g.; asparagine 0.5 g.; K_2HPO_4 , 0.5 g.; distilled water, 1 l. The solution was adjusted to pH 7.0–7.2 and sterilized by steaming for 20 min. on 3 consecutive days. Buffered glucose asparagine solution was made by mixing equal volumes of the phosphate buffer solution used for plain buffered agar medium and double-strength glucose asparagine solution from which the phosphate had been omitted. This solution was also sterilized by intermittent steaming.

Other materials

Leaf mould. This was washed as free as possible from mineral particles in a column of water agitated by a stream of air (Chesters, 1947).

Root fraction. Soil from the top 6 in. of local pasture was washed on a wire-mesh sieve to separate the larger pieces of fresh and partially decomposed organic matter. This material (consisting mainly of fine roots) was washed in the same way as the leaf mould.

Leaf mould, root fraction and other organic materials (dried grass, wheat straw, farmyard manure, horticultural peat) were all dried at 50°, milled and sifted. Fractions passing a 0.5 mm. sieve were used.

RESULTS

Inhibition of fungal growth by actinomycetes on agar media containing different concentrations of glucose

Three media were prepared from the AS solution solidified with 1.5% (w/v) agar. Medium A contained no glucose; medium C, 0.1 g. glucose/l., and medium E, 10 g. glucose/l. Five fungi were tested against five actinomycetes on each medium in Petri dish cultures. Three actinomycete species were tested in each dish, the inocula being placed as spots near the edge of the dish. After 5 days

of incubation at 25°, a small spot inoculum of the test fungus was placed in the centre; control plates with fungi alone were also set up. The cultures were incubated and daily measurements were made of (1) the distance grown by each fungus from the centre towards each actinomycete colony; (2) the distances which separated fungus and actinomycete colonies along lines joining their centres.

Table 1. *Distances grown by fungi towards actinomycete colonies after 9 days of incubation*

		Medium: AS solution. (1) Distances grown (mm.). (2) Distances between fungus and actinomycete colonies					
Fungus	Actino- mycete	No glucose		0.01 % glucose		1 % glucose	
		(1)	(2)	(1)	(2)	(1)	(2)
<i>Fusarium culmorum</i>	A2	24.5	0	23.0	1.0	16.0	9.0
	A30	24.5	0	23.0	0	14.5	9.0
	A40	23.5	0	18.0	4.0	18.0	5.0
	S.a.	20.0	4.0	16.5	7.0	17.5	6.5
	S.l.	23.0	0	18.0	3.0	16.5	5.0
	.	44.0+	.	44.0+	.	44.0+	.
<i>Fusarium</i> sp.	A2	25.0	0	23.5	1.0	13.5	11.0
	A30	24.0	0	23.0	0	12.0	11.5
	A40	22.0	0	22.0	0	18.0	4.0
	S.a.	.	.	18.5	5.0	15.5	7.5
	S.l.	21.5	0	20.0	0	16.0	5.5
	.	35.8	.	37.0	.	32.8	.
<i>Mycelia sterilia</i>	A2	18.0	6.0	11.0	9.0	9.0	15.0
	A30	22.0	1.0	19.0	4.0	9.0	14.0
	A40	23.5	0	14.5	7.0	12.5	9.0
	S.a.	20.5	3.0	19.0	4.5	13.5	9.0
	S.l.	21.0	0	17.5	3.5	14.5	6.0
	.	25.9	.	24.1	.	27.0	.
<i>Stemphylium</i> sp.	A2	20.0	4.5	15.5	8.5	9.5	15.5
	A30	23.5	0	20.0	3.0	8.0	15.0
	A40	23.0	0	18.5	4.5	14.5	6.0
	S.a.	21.0	3.0	19.0	5.0	15.5	7.5
	S.l.	22.0	0	21.0	0	17.0	5.0
	.	40.0	.	34.3	.	39.5	.
<i>Rhizoctonia goodyerae-repentis</i>	A2	20.0	4.5	.	.	17.0	8.0
	A30	24.0	0	.	.	8.0	15.0
	A40	23.0	0	17.0	5.5	16.0	6.0
	S.a.	24.0	0	.	.	14.0	9.0
	S.l.	22.0	0	17.5	2.5	7.0	6.5
	.	40.5	.	44.0+	.	41.0	.

S.a. = *Streptomyces albidoflavus*; S.l. = *S. lavendulae*.

Measurements of fungal growth after 9 days are given in Table 1. The inhibitory effect of the actinomycetes on the fungi in all combinations was directly related to the glucose supply. The fungi reached the actinomycetes within 9 days in 17 out of the 25 combinations on medium A, in only 5 combinations with medium C, and in no case with medium E. After 23 days of incubation

the fungi reached the actinomycetes in all but one combination on media A and C and in only 4 combinations on medium E. Only on medium E did the actinomycetes virtually arrest fungal extension.

Antagonism in sand cultures with artificial soil solution

Various flask culture experiments were made in order to study the behaviour of an actinomycete and a fungus when growing together under conditions approximating more closely to those in soil. *Streptomyces albidoflavus* was used with the fungus *Fusarium culmorum*. Ten g. of coarse, washed and ignited sand were placed in a 50 ml. conical flask which was then plugged and autoclaved for 1 hr. at 15 lb./sq.in. After sterilization, each flask received 1–2 ml. of culture solution containing the inoculum. The contents of each flask were then mixed thoroughly by shaking.

Sampling and estimation of fungal growth. About 1 g. of sand was removed from the flask with a flamed spatula and transferred to a weighed crucible. One ml. of water was added, the sample ground with the end of a glass rod for 5 min. and 0.5 ml. of the resulting suspension mixed with an equal volume of acetic acid-aniline blue solution (Jones & Mollison, 1948). A drop of this mixture was placed on a haemocytometer of 0.1 mm. depth and the number of stained fragments counted; a $\frac{2}{3}$ in. objective was used. The number of hyphal fragments/ml. suspension was estimated from the mean count for 10 random microscope fields on each of 4 replicate slides. This value was corrected for moisture to give the number of fragments/g. of culture.

AS solution E (10 g. glucose/l.) was used to moisten the sand in the culture flasks. Suspensions of spores of both organisms were made in this medium. Three sets of sand flasks (2 flasks/set) received the following additions: *Set A.* 1 ml. fungus spore suspension + 1 ml. medium. *Set B.* 1 ml. fungus spore suspension + 1 ml. actinomycete spore suspension. *Set C.* 1 ml. actinomycete spore suspension + 1 ml. medium. After 5 days of incubation at 25°, each flask in set C received 1 ml. of fungus spore suspension. The flasks were sampled after a further 2 and 7 days of incubation and the number of fragments of fungus mycelium/g. dry wt. culture estimated as described above. Since fungus growth in duplicate flasks was obviously similar, quantitative estimations were made on only one flask of each set (A1, B1 and C1). In flask B1 the fungus grew almost as well as it did in the control flask A1 (Table 2), and was accompanied by only slight development of the actinomycete. There was no evidence of any antibiotic action in flask B1, in which both organisms had been present for the same length of time, so it was discarded after 7 days.

In flask C1 the actinomycete grew only very slowly during the 5 days which preceded inoculation with the fungus. After inoculation the fungus at first grew rapidly (thereby showing that no effective concentration of antibiotic had previously been built up) but later the amount of detectable fungus mycelium in the culture declined. This decline coincided with marked development of the actinomycete. Seven days after fungus inoculation, the actinomycete was growing on all the few remaining recognizable fragments of fungus

mycelium. The nature of this direct attack on the fungus is illustrated in Pl. 1, figs. 1, 2. After 15 days, all trace of fungus growth had disappeared. At this time, both C cultures were tested for glucose (by Fehling's solution), nitrate (by Gries-Ilosvay reagent) and phosphate (by the benzidine-blue spot test; Feigl, 1943). Both glucose and nitrate were present but no positive test was obtained for phosphate.

Table 2. *Growth of Fusarium culmorum with Streptomyces albidoflavus in sand moistened with AS solution containing 10 g. glucose/l.*

	Incubation after inoculation with fungus for (days)	
	2	7
A 1. Control (no actinomycete)	46.7	299.9
B 1. Fungus and actinomycete inoculated simultaneously	39.5	240.5
C 1. Fungus inoculated 5 days after the actinomycete	91.1*	3.5

* This figure is probably high because each C flask received 3 ml. medium (2 ml. initially and 1 ml. with the fungus inoculum), whereas each A and B culture received only 2 ml.

Antagonism in sand cultures with actinomycete and fungus inocula of different strengths

An experiment was set up to determine whether antibiotic effects would become apparent and direct-attack effects modified by changes in the numbers of fungus and actinomycete spores present in the original inocula.

A suspension of spores of *Streptomyces albidoflavus* in sterile 0.75% (w/v) NaCl solution was freed from particles of mycelium by filtration through a sterile no. 1 filter-paper and 0.1 ml. of the filtered suspension was mixed with an equal volume of acetic acid-aniline blue stain and allowed to stand for 10 min. The number of stained spores/ml. was then estimated microscopically with a Thoma haemocytometer and oil-immersion objective. Appropriate dilutions were made from this original suspension. The number of spores of *Fusarium culmorum*/ml. was estimated directly with a haemocytometer and dilutions prepared.

Each of twenty sand culture flasks received 2 ml. of AS solution E. These 2 ml. portions were made up by combining appropriate volumes of standardized suspensions of spores of both organisms prepared with the AS solution. Sterile medium was added to the flasks as required to bring the total volume of liquid added to each flask to 2 ml. A small sample was removed daily from each flask and mixed with a little acetic acid-aniline blue solution. Each resulting suspension was examined microscopically (Table 3).

Similar results were obtained with either 10^3 or 10^4 fungus spores/flask; only those results obtained with 10^3 spores/flask are therefore given. In all the test cultures, the fungus developed rapidly at first and actinomycete growth

was delayed. Nevertheless, by 22 days, the actinomycete had in all cases attacked the fungus directly. Clearly, the faster growing fungus had a great initial advantage over the actinomycete, and this situation was not affected very much by the numbers of actinomycete and fungus spores which comprised the inocula. It was clear from this and the preceding experiment that

Table 3. *Growth of Fusarium culmorum with Streptomyces albidoflavus in sand moistened with ASP solution containing 10 g. glucose/l.*

Actinomycete spores (no./flask)	Actinomycete (amount of growth); days of incubation			Fungus (kind of mycelium); days of incubation		
	7	12	22	7	12	22
0 (Control)	—	—	—	N	N	N
5×10^3	—	+	++	N	N	N
5×10^4	—	++	+++	N	U	O
5×10^5	—	++	+++	N	U	O
5×10^6	—	++	+++	N	U	O

— = no growth; + = slight growth; ++ = moderate growth; +++ = abundant growth; N = normal deeply stainable mycelium; U = largely unstainable mycelium; O = no stainable mycelium.

any demonstration of early antagonistic effects in sand culture (as distinct from the direct attack which always took place when the fungus grew before the actinomycete) depended on the actinomycete being able to grow and produce its antibiotic before the fungus inoculation. When using AS solution E, the actinomycete did not grow sufficiently rapidly to produce an effective concentration of the antibiotic even when inoculated into the culture 5 days before the fungus; it was thought that phosphate deficiency might be responsible for this slow actinomycete growth even though satisfactory growth of this organism and other actinomycetes had taken place on the same medium solidified with agar.

A test was made by growing the actinomycete in small quantities of (a) AS solution E as used above, and (b) the same medium in which the concentration of K_2HPO_4 had been raised to 0.5 g./l. This test showed that the actinomycete was able to grow much faster in the latter medium than in the former. Luxuriant actinomycete development took place in the medium with high phosphate within 5 days, whereas practically no growth occurred in the unmodified medium in this time. In all subsequent experiments, ASP solution refers to AS solution modified with the extra phosphate.

Effect of glucose concentration of the inhibition of Fusarium culmorum by Streptomyces albidoflavus in sand culture with ASP solution

Eight sand culture flasks each received 1 ml. of ASP solution containing 0.1 g. glucose/l.; eight more received 1 ml. ASP solution containing 10 g. glucose/l. Four flasks of each set were inoculated with actinomycete spores and incubated for 5 days at 25°. Each flask was then inoculated with spores of

Fusarium culmorum in 1 ml. of medium. The amount of fungus growth was measured after 1 and 2 days of further incubation (Table 4). At the higher glucose concentration the growth of the fungus was greatly inhibited in the presence of the actinomycete. Two days after the fungus inoculation, glucose was present in abundance, and nitrate and phosphate were also present in these mixed cultures. The antagonism, therefore, may have been due to antibiotic action or to competition for some nutrient other than those named above. At the lower glucose concentration very slight inhibition of the fungus occurred. It was not clear whether this slight inhibition should be ascribed to antibiosis or to competition between the organisms for some limiting nutrient, e.g. the small amount of glucose in the culture flasks (c. 0.2 mg./flask).

Table 4. *Inhibition of growth of Fusarium culmorum by Streptomyces albidoflavus in sand moistened with ASP solution containing glucose*

Conc. of glucose in liquid medium (%, w/v)	Type of culture	Period of incubation after inoculation with fungus (days)	
		1	2
		No. fragments fungus mycelium ($\times 10^3$)/g. dry wt. culture	
0.01	Fungus alone	29.7	48.2
	Fungus + actinomycete	24.3	35.7
1.0	Fungus alone	31.1	236.8
	Fungus + actinomycete	1.9	3.6

Under certain conditions, growth of the fungus spores was completely inhibited in a way that cast no doubt on the production of antibiotic by the actinomycete. Two sand flasks each received 1 ml. of glucose asparagine solution (with the same concentrations of glucose and phosphate as the ASP solution with 10 g. glucose/l.) containing spores of the actinomycete. After incubation for 5 days each flask received 1 ml. of medium containing spores of *Fusarium culmorum*. Two control flasks, which had not previously been inoculated with the actinomycete, were also inoculated with the fungus. In the control cultures, fungus spores began to germinate within 3 hr. but, in the presence of the actinomycete, no germination was seen even after 23 hr. and the spores remained unswollen and lost their refractility as they do in the presence of active actinomycete secretions in liquid or agar media. It seemed unlikely that germination had been suppressed by lack of oxygen caused by the growing actinomycete since fungus spores germinated satisfactorily on glucose asparagine agar slopes in sealed tubes in which the oxygen tension had been drastically lowered by alkaline pyrogallol. Also, spores germinated overnight in distilled water so deficiency of nutrients could not have caused failure of germination. Whenever germination of fungus spores is complete, as in this experiment, the production of antibiotic by the actinomycete is certain but when germination takes place and is followed by some suppression of further fungus growth it is difficult to decide how much this suppression is due

to antibiotic actinomycete secretions and how much to competition between the organisms for nutrients. This question will be examined in a later communication.

Inhibition of Fusarium culmorum by Streptomyces albidoflavus with natural organic materials as nutrient sources

To gain some idea of the type of organic matter likely to support growth of the fungus and the actinomycete and to permit antibiotic production by the latter, several natural organic materials, each likely to occur in soils, were tested. Dried grass, wheat straw, leaf mould, root fraction, farmyard manure, peat and lignin were tested by the following method, which was used because it enabled the pH values of the cultures to be kept constant. Thirty-two Petri dishes each containing 15 ml. plain buffered agar medium (pH 7.0) were left overnight at room temperature to allow the agar surfaces to dry. Next day, a straight line with its centre point *c.* 30 mm. from the circumference was drawn in indian ink across the bottom of each dish (the 'base-line'). Sixteen plates (test plates) were then streaked with a suspension of actinomycete spores in such a way that the edge of the streak facing the centre of the plate was immediately above the base-line. When the streaks were dry, the plates were supplied with the organic materials. The material to be tested (finely milled and sterilized) was deposited with a flamed spatula over the whole area between the base-line and the nearer part of the plate circumference. The sixteen uninoculated (control) plates received organic matter in the same way; each test was made in duplicate. Two mixed culture and two control plates received no addition of organic material.

After 5 days of incubation, a suspension of *Fusarium culmorum* spores was streaked on each plate in a line parallel with the base-line and 30 mm. from it. Organic matter (of the same kind as that already present on the plate) was then distributed between the fungus streak and the rim of the dish to provide a source of nutrients for the fungus (Pl. 2). In this way, solid material was provided for both organisms, while leaving the space between them clear of organic particles which would otherwise interfere with microscopic examination and the measurement of fungus growth. Daily measurements of the distance grown by the fungus toward the actinomycete in each mixed culture, and the distance grown toward the base-line in each control culture, are given in Table 5.

Fungal growth towards the actinomycete was virtually arrested after 5 days in the dried grass cultures. Considerable distortion of the marginal fungal hyphae suggested that the inhibition was caused by antibiotic secretions from the actinomycete. Inhibition also took place on the plain buffered agar test plates though this was not sufficient to arrest growth of the fungus or to prevent contact between the organisms after 9 days of incubation. Though this medium was poor in nutrients, extension of the fungus in the corresponding control cultures was rapid though the amount of mycelium formed was obviously very small compared with that in the dried grass controls. It was therefore assumed that the actinomycete produced the antibiotic on the plain

buffered agar. With the remaining materials tested the fungus grew until it approached closely to the actinomycete and inhibition was less than on plain agar. These materials had thus actually decreased the inhibition of fungal growth by the actinomycete, although the latter grew more strongly than in the absence of these materials. This suggested that all the materials except dried grass were able to inactivate to some extent any antibiotic formed from the plain agar base.

Table 5. *Distance grown by Fusarium culmorum toward Streptomyces albidoflavus on buffered agar with various solid organic materials*

Organic material	Inoculum	Period of incubation after inoculation with fungus (days)		
		5	6	7
		Distance grown by <i>F. culmorum</i> towards <i>S. albidoflavus</i> (mm.)		
Dried grass	F + A	13.2	13.5	14.5
	F	24.9	30.0 +	
Wheat straw	F + A	19.8	23.5	25.6 C
	F	23.8	30.0 +	
Root fraction	F + A	22.1	25.7	26.9 C
	F	24.2	30.0 +	
Leaf mould	F + A	20.1	24.1	26.6 C
	F	25.0	30.0 +	
Farmyard manure	F + A	20.7	24.1	26.2 C
	F	22.0	30.0 +	
Peat	F + A	18.7	21.5	24.4 C
	F	21.7	29.4	30.0 +
Lignin	F + A	17.2	19.8	22.6
	F	20.9	28.4	30.0 +
None (plain buffered agar alone)	F + A	16.5	18.9	21.1
	F	20.2	26.7	30.0 +

F = fungus alone; F + A = fungus + actinomycete; 30.0 + = growth of fungus beyond base-line; C = contact made between organisms.

*Inactivation by organic materials of the antibiotic formed
by Streptomyces albidoflavus*

In order to determine the direct effects of some of the natural soil materials used on the antibiotic of *Streptomyces albidoflavus*, an experiment was made with sterile filtrates of liquid cultures of this actinomycete. *S. albidoflavus* was cultivated in glucose asparagine solution (buffered at pH 7.0) for 7–10 days at 25° and a filtrate was prepared by passing this culture through a no. 5 (or no. 50) filter-paper contained in a sterile filtration assembly (Fig. 1). These filtrates usually remained sterile though actinomycete spores sometimes passed through the filter. To prevent renewed actinomycete growth, each filtrate was heated in boiling water for 2 min., a process which decreased its antibiotic activity only slightly.

Ten mg. or 100 mg. portions of dried sterile materials (dried grass, leaf

mould, root fraction and peat) were placed in test tubes. Each tube then received 5 ml. of *Streptomyces albidoflavus* culture filtrate, 1 ml. of fresh medium and an inoculum of *Fusarium culmorum* spores. Control tubes without the organic materials were also set up. Another set of tubes each received 5 ml. of *S. albidoflavus* filtrate, 1 ml. of extract prepared from either 10 mg. or 100 mg. of organic matter/ml. of fresh extracting medium, and the inoculum.

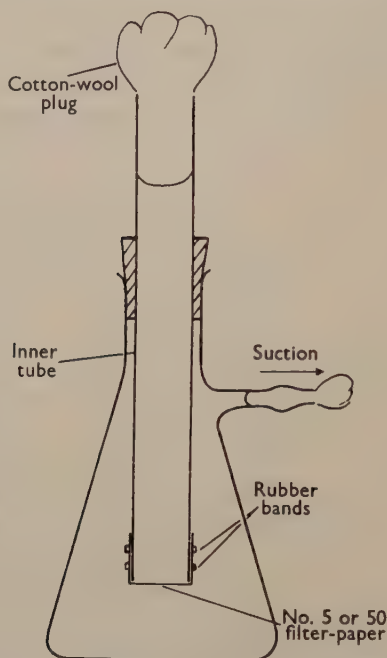


Fig. 1. Apparatus for filtration under sterile conditions. Solutions to be filtered are introduced into inner tube.

The filtrate of *Streptomyces albidoflavus* was able to prevent fungal growth in the controls, but the presence of each of the natural materials in solid form enabled *Fusarium culmorum* to grow. Even extracts of the materials had some effect in neutralizing antibiotic action. This effect varied with the nature of the material, being greatest with root fraction and least with dried grass and peat. This effect seems to be due to the presence of substances which either enable the fungus to overcome the effects of the antibiotic or which destroy it directly. It was interesting to note that dried grass which favoured antibiotic production by *S. albidoflavus* on solid agar medium also contained substances which could, to some extent, inactivate that antibiotic.

DISCUSSION

The experiments with *Streptomyces albidoflavus* and *Fusarium culmorum* growing together in sand suggest that the actinomycete antagonizes the fungus in at least two ways. When actinomycete growth was at first poor in media low

in phosphate the fungus was able to make some mycelial growth before antagonism became noticeable. After a while, however, the actinomycete attacked and grew at the expense of the fungus mycelium. This attack took place under all conditions that allowed fungus mycelium to develop in the mixed cultures. But, where the actinomycete made good growth before the introduction of the fungus it was able to check the latter at an early stage. It remains a question how far this early check was due to antibiotic action or to competition for some limiting nutrient. Early antagonism was intensified by increasing the glucose and phosphate concentration which also increased early growth of the actinomycete. That this effect was at least in part due to antibiotic action is suggested by the observed inhibition of fungus spore germination under these conditions. Moreover, the fact that higher glucose concentration similarly increased the antagonism of the actinomycetes to fungi at a distance on agar plates supports this conclusion.

In natural soil the supply of easily available nutrients is limited and it is an important question whether the nature of these materials is such as to permit actinomycetes to grow and produce antibiotics in sufficient quantities to be effective. Experiments with some natural materials of a type likely to be found in soils showed that dried grass at least was able to support actinomycete growth and antibiotic production, but, on the other hand, that all the materials tested had some effect in inactivating the antibiotics produced. This raises the further question of how far these and other soil constituents can inactivate actinomycete antibiotics and, where they do so, how much antagonism can be exerted by the actinomycete through other means such as competition for limiting nutrients. This question will be considered in a further communication. The present results emphasize one point to which little attention has hitherto been given. This is that when supplies of carbon and energy sources are low, conditions may be unfavourable not only for growth of a potential antibiotic producer but also for that of other micro-organisms susceptible to the antibiotic when produced. The relative importance of such growth-limiting mechanisms as antibiotic production, nutrient competition, and direct attack by one organism on another at low nutrient concentrations has been scarcely studied as yet, and more information on this subject is needed if the complex interactions affecting survival of micro-organisms in soils are to be understood.

This paper is part of a thesis accepted for the degree of Ph.D. at the University of London, 1953. I wish to thank Dr H. G. Thornton, F.R.S., for his helpful advice throughout the course of this work, Miss Angela Roe for technical assistance and Miss Mabel Dunkley for preparing the typescript. I am also indebted to the Agricultural Research Council for a grant which made possible the initiation of this research.

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EXPLANATION OF PLATES

PLATE 1

Streptomyces albidoflavus and *Fusarium culmorum* growing in sand moistened with ASP solution containing 10 g. glucose/l. Fungus inoculated 7 days after actinomycete.

Fig. 1. Two days after inoculation with fungus. Actinomycete beginning to attack fungus mycelium.

Fig. 2. Ten days after inoculation with fungus. Actinomycete growing on fungus mycelium which has lost its staining property. Water mounts. Stained with acetic acid-aniline blue. Magnification $\times 296$.

PLATE 2

Growth of *Streptomyces albidoflavus* and *Fusarium culmorum* on leaf mould distributed on a buffered agar surface. Fungus growth too transparent to be visible in photograph.

(Received 27 September 1955)

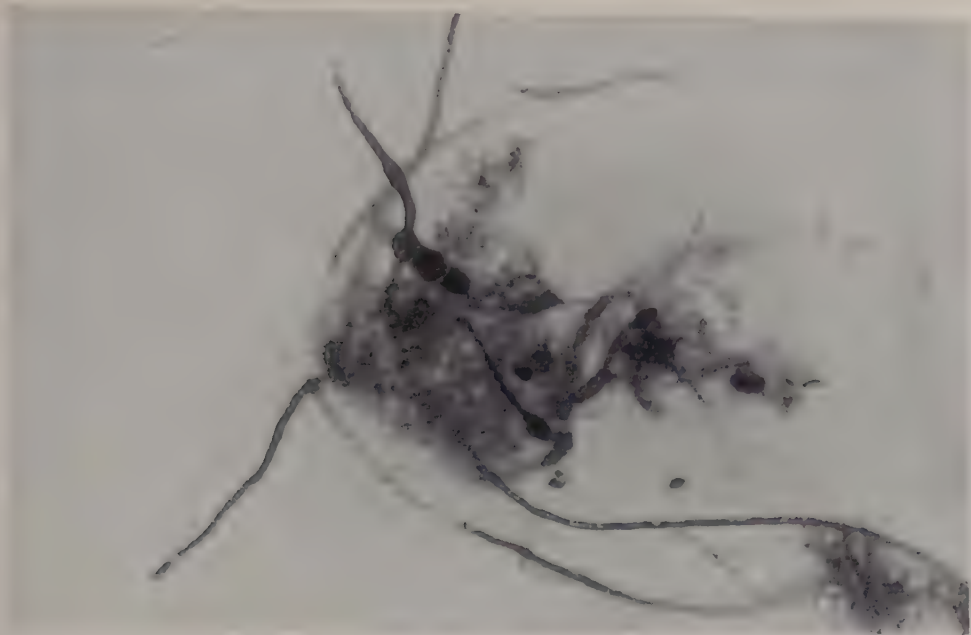


Fig. 1

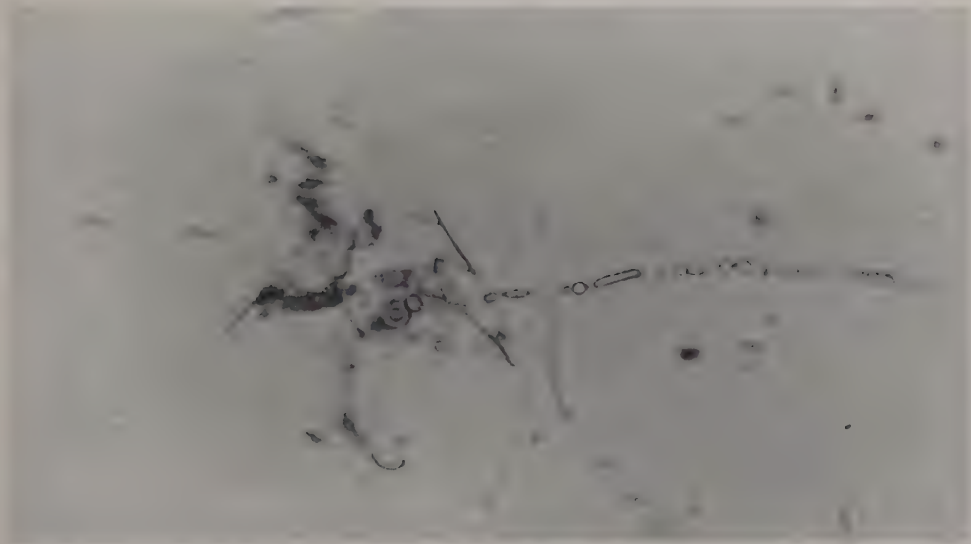
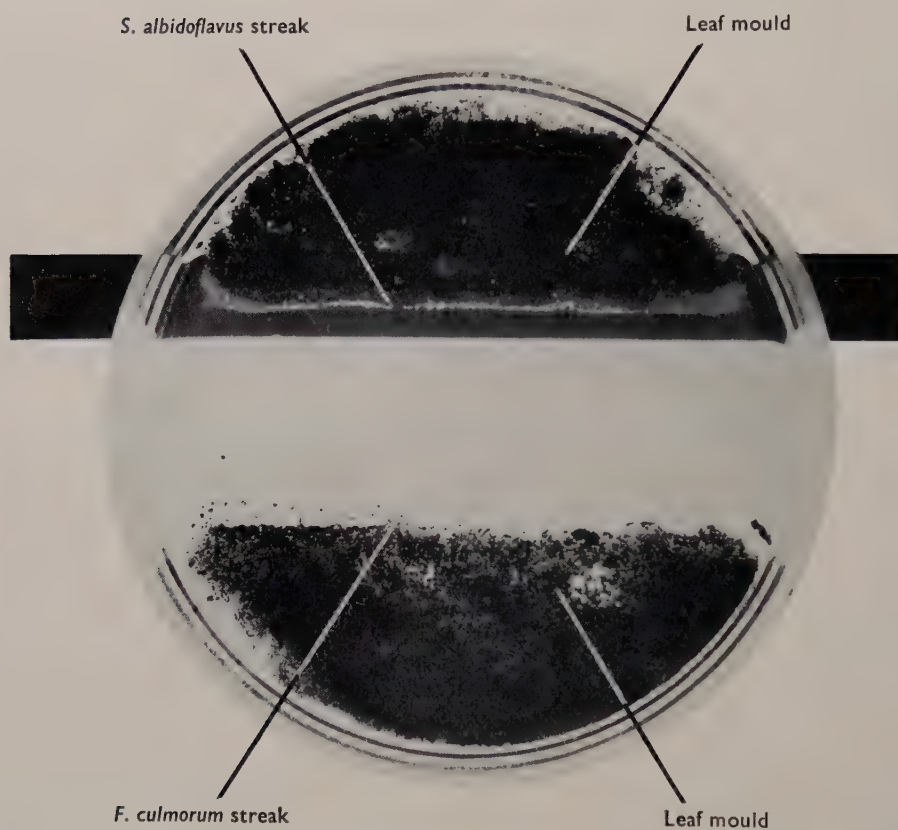


Fig. 2

F. A. SKINNER—ANTIFUNGAL ACTIVITIES OF SOIL ACTINOMYCETES. PLATE 1

(Facing p. 392)



The Effect of Adding Clays to Mixed Cultures of *Streptomyces albidoflavus* and *Fusarium culmorum*

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SUMMARY: An antibiotic present in culture filtrates of *Streptomyces albidoflavus* was inactivated by clays and by suspensions and extracts of soils. When this actinomycete was grown with *Fusarium culmorum* in a sand + bentonite mixture moistened with nutrient solution, it did not antagonize the fungus by antibiotic secretions. However, suppression of fungus growth was observed even in the presence of bentonite particularly when glucose was present in abundance; this effect was attributed to competition between the organisms for limiting nutrients. The actinomycete also lysed the contents of the fungus mycelium in sand culture but not when bentonite was added. The lytic agent appeared to differ from the antibiotic. Neither antibiotic action nor direct (lytic) attack on the fungus was demonstrated in sterilized soil.

In a previous paper (Skinner, 1956) the complexity of the relationship between *Streptomyces albidoflavus* and *Fusarium culmorum* was indicated. In sand culture the growth of the fungus was suppressed by antibiotic secretions of the actinomycete and preformed fungus mycelium was destroyed. Even when glucose concentrations were low, some inhibition of fungus growth by the actinomycete was observable, but it was not clear whether this was due to the presence of antibiotic substances or to competition for limiting nutrients. There are many factors in sterile and in unsterile soil which tend to inactivate antibiotics which have been added or formed *in situ* (Brian, 1949; Siminoff & Gottlieb, 1951; Gottlieb & Siminoff, 1952; Jefferys, 1952). In particular, clays frequently adsorb basic antibiotics which are elaborated by the majority of antibiotic-producing *Streptomyces* spp., and render them inactive. In view of this it was considered that the inclusion of clays in mixed sand cultures might well eliminate antibiotic effects and so permit some distinction to be made between (a) inhibition of growth of *Fusarium culmorum* caused by antibiotic actinomycete secretions; (b) limitation of growth of this fungus because of nutrient deficiencies brought about by growth of the actinomycete.

The micro-organisms, materials and methods were generally the same as described in the preceding paper (Skinner, 1956); techniques peculiar to this paper are described at appropriate places in the text.

RESULTS

Inactivation of the antibiotic formed by Streptomyces albidoflavus in culture filtrates

Test-tubes, each containing 5 ml. of glucose asparagine solution buffered at pH 7.0 (M/15 KH_2PO_4 soln., 195 ml.; M/15 Na_2HPO_4 soln., 305 ml.; glucose, 10 g.; asparagine 0.5 g.; distilled water to 1 l.) were sterilized by steaming for

20 min. on each of three consecutive days, inoculated with spores of *Streptomyces albidoflavus* and incubated at 25°. After 8 days, the cultures were divided into two sets. One set remained untreated (untreated tubes). The contents of tubes of the second set were bulked, filtered through a sterile no. 5 filter-paper and the filtrate redistributed in 5 ml. portions into sterile tubes. These tubes were then immersed in boiling water for 2 min., and then cooled rapidly in cold water (filtrate tubes). Duplicate tubes of each set then received sterile additions of bentonite or kaolin suspended in 1 ml. fresh medium (see Table 1). Control tubes did not receive the clays. Each tube was then inoculated with spores of *Fusarium culmorum* and incubated.

Table 1. *Growth of Fusarium culmorum in liquid cultures and culture filtrates of Streptomyces albidoflavus*

Material added	Amount added/tube (mg.)	Kind of culture	Relative amount of fungal growth		
			Period of incubation (days)		
			2	7	16
Control (no addition)	—	Untreated	—	—	—
		Filtrate	—	—	—
Bentonite	1	Untreated	++	++	++
		Filtrate	++	++	++
	10	Untreated	++	++	++
		Filtrate	++	++	++
Kaolin	10	Untreated	—	—	—
		Filtrate	—	+	++
	100	Untreated	—	—	—
		Filtrate	—	++	++

— = no growth of fungus; + = trace of fungal growth; ++ = good growth of fungus (mycelium occupying at least $\frac{1}{4}$ volume of culture).

During the first 2 days the fungus grew only in tubes containing bentonite. Fungus growth appeared in the filtrate tubes without clay after 3 weeks; this was attributed to decay of the inhibitor. The fungus did not grow in the untreated controls; in this set the concentration of antibiotic was presumably maintained by continued growth of the actinomycete. The fungus began to grow between the 2nd and 7th days in filtrate tubes which contained kaolin. Probably the kaolin adsorbed some antibiotic, but not enough to decrease the initial concentration quickly to a value at which fungus growth could begin. However, this critical concentration was apparently reached by decay of the remaining unadsorbed antibiotic after a short time.

Bentonite (1 mg.) also inactivated the antibiotic produced in 5 ml. of ASP solution E containing 10 g. glucose/l. (see p. 395).

Two Rothamsted clays (one derived from soil of Broadbalk field plot 3, and the other from soil of the meteorological enclosure) and three soils (Broadbalk plot 2, and an Australian chernozem and laterite) also inactivated the antibiotic when tested in the same way at rates of 10 mg. and 100 mg./tube of 5 ml. of

liquid culture or culture filtrate, though none of these was nearly so effective as bentonite. Besides the tubes which received suspensions of the three soils, duplicate sets of tubes received 1 ml. portions of centrifuged sterile extracts prepared from 10 mg. or 100 mg. of soil/ml. extracting glucose asparagine solution. These extracts were also effective in inactivating the antibiotic, though generally to a lesser degree than the corresponding suspensions. The activity of these extracts was probably due to the organic matter in the soils (Skinner, 1956). The effectiveness of bentonite in inactivating the antibiotic suggested that bentonite would be a suitable material for adding to mixed cultures of the two organisms in order to eliminate antibiotic effects.

Flask culture experiments

Sand and sand + clay mixtures in flasks were moistened with an artificial soil solution supplemented with different concentrations of glucose. The artificial soil solution (hereafter ASP solution) had the following composition (g./l. distilled water): CaSO_4 , 0.8; $\text{Ca}(\text{NO}_3)_2$, 0.33; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.7; K_2SO_4 , 0.025; K_2HPO_4 , 0.5; NaHCO_3 , 0.2; FeCl_3 , trace. This solution, with glucose additions, was sterilized by steaming for 20 min. on three consecutive days; the pH value was adjusted to 7.0–7.2.

An experiment was made to determine whether *Streptomyces albidoflavus* would inhibit the growth of *Fusarium culmorum* in sand cultures containing clays or silt. Nine sets of 50 ml. flasks (4 flasks/set) were prepared. The flasks of each set contained one of the following mixtures: (1) 10 g. sand; (2) 9.9 g. sand + 0.1 g. bentonite; (3) 9 g. sand + 1 g. bentonite; (4) 9.9 g. sand + 0.1 g. kaolin; (5) 9 g. sand + 1 g. kaolin; (6) 9.9 g. sand + 0.1 g. Broadbalk clay; (7) 9 g. sand + 1 g. Broadbalk clay; (8) 9.9 g. sand + 0.1 g. Broadbalk silt; (9) 9 g. sand + 1 g. Broadbalk silt. Two flasks of each set received spores of *Streptomyces albidoflavus* suspended in 1 ml. of ASP solution containing 10 g. glucose/l.; and were incubated at 25°. The remaining two flasks of each set (controls) were not inoculated. After 5 days of incubation each flask was inoculated with spores of *Fusarium culmorum* suspended in 1 ml. of medium. The incubation of the cultures was then continued and the flasks were examined at intervals.

The actinomycete completely inhibited fungus growth in sets 1, 8 and 9 which had no additions of clays, but did not do so in the flasks of the remaining sets. In most of these clay cultures, the fungus grew to some extent after only 1 day of incubation. It was, however, obvious that fungus mycelium in the presence of the actinomycete in these clay cultures was not nearly so abundant as it was in the corresponding control cultures without the actinomycete.

Quantitative measurements were made of fungus growth in sand cultures set up as in the foregoing experiment. Two sets of culture flasks were prepared; those of one set each contained 10 g. of sand; those of the other set, 10 g. of sand + bentonite mixture (100 mg. bentonite). Three replicates of each set were incubated at 25° and one of them was used for each sampling. During incubation each flask received 0.5 ml. sterile water weekly to make up

evaporation loss. Estimates of numbers of fragments of fungus mycelium and spores were made by the method already described (Skinner, 1956). The actinomycete completely inhibited fungus growth in cultures without bentonite and attacked the spores added as inoculum (Table 2). These spores became enveloped in actinomycete mycelium and rapidly lost their staining properties. In the older cultures fungus spores were not detected, either because they had

Table 2. *Growth of Fusarium culmorum with Streptomyces albidoflavus in sand and sand+bentonite moistened with ASP solution containing 10 g. glucose/l.*

		No. fungal spores and hyphal fragments ($\times 10^3$)/g. dry wt. culture					
		Period of incubation after inoculation with fungus (days)					
		3		11		32	
		Spores	Hyphae	Spores	Hyphae	Spores	Hyphae
Sand	<i>F. culmorum</i> alone	23.7	184.8	105.4	250.2	178.9	335.2
	<i>F. culmorum</i> + actinomycete	1.24*	0	0.38†	0	0	0
Sand + bentonite (1 %, w/w)	<i>F. culmorum</i> alone	41.2	163.2	150.4	307.1	273.7	272.7
	<i>F. culmorum</i> + actinomycete	0	20.1	1.1	86.9	8.1	143.5

* Spores introduced as inoculum.

† Direct actinomycete attack on fungus spores.

been completely destroyed or were hidden in the mass of actinomycete mycelium. Spores were not attacked in this way when bentonite was present. However, the fungus made much less growth in the presence of the actinomycete, even in the flasks containing 100 mg. bentonite. Since 1 mg. bentonite inactivated the antibiotic produced in 5 ml. liquid, actinomycete culture or heated culture filtrate, prepared either with buffered glucose asparagine solution or with ASP solution E, it may be assumed that the 100 mg. bentonite present in the 10 g. sand + bentonite cultures was more than adequate to inactivate any antibiotic formed from the 2 ml. of ASP solution contained in such cultures. It is therefore reasonable to attribute the suppression of fungus growth in such cultures (as compared with the fungus growth in the sand + bentonite control cultures without the actinomycete) to competition between the two organisms for some limiting nutrient.

It might be objected that an antibiotic may be adsorbed on a colloid yet still retain some activity. This was found by Pramer & Starkey (1950), who observed that when soil which had adsorbed streptomycin was added to agar medium, the antibiotic still exerted its effects on bacteria inoculated on to the agar surface. Thus, it would be expected that micro-organisms in soil in direct contact with particles with adsorbed streptomycin would be influenced by this antibiotic.

An experiment was made to determine whether the *Streptomyces albidoflavus* antibiotic remained similarly active when adsorbed on bentonite. Plates of ASP solution agar containing 1.5 % (w/v) bentonite were streak-inoculated with spores of *S. albidoflavus*, incubated at 25° for 5 days and then inoculated with *Fusarium culmorum* at a distance of 3 cm. from the actinomycete growth. On these plates the fungus grew steadily towards the actinomycete and made contact with it within 7 days, whereas on similar plates without bentonite fungus growth was arrested at a distance from the actinomycete. Similar results were obtained on glucose asparagine agar with and without bentonite. In these cultures, the fungus grew well in intimate contact with particles of bentonite which must have adsorbed the antibiotic. Thus the evidence was against the possibility that bentonite charged with the *Streptomyces albidoflavus* antibiotic would suppress growth of *Fusarium culmorum* in contact with it.

Inhibition of fungus growth in sand and sand + bentonite cultures in media with low glucose concentrations

It had been found that the growth of *Fusarium culmorum* was inhibited slightly, even in sand moistened with medium containing low concentrations of glucose (Skinner, 1956). An experiment was therefore made to determine whether inhibition at such low glucose concentrations might be attributed to antibiotic action. Flask cultures containing sand or sand + bentonite mixture were prepared and inoculated with *Streptomyces albidoflavus* spores contained

Table 3. *Growth of Fusarium culmorum with Streptomyces albidoflavus in sand and sand + bentonite moistened with ASP solution*

Medium	Type of culture	Inoculum		Ratio control: mixed culture
		Actino- mycete + fungus (No. fragments mycelium ($\times 10^3$)/g. dry wt. culture)	Control (fungus alone) fungus	
B (0.001 % glucose)	Sand	7.4	26.9	3.64
	Sand + bentonite	11.9	19.5	1.64
C (0.01 % glucose)	Sand	9.4	41.2	4.38
	Sand + bentonite	25.6	28.9	1.13
D (0.1 % glucose)	Sand	6.2	79.2	12.77
	Sand + bentonite	12.4	66.5	5.36

in 1 ml. portions of ASP solution media B, C and D containing respectively 0.01, 0.1. and 1.0 g. glucose/l. The flasks were incubated for 5 days before inoculation with *F. culmorum* spores. The amounts of fungus mycelium were estimated after a further day of incubation. Some inhibition of fungus growth took place in all cultures containing the actinomycete (Table 3), although bentonite again decreased the degree of inhibition (as indicated by the ratio of control: test estimates).

In media B and C (low glucose concentrations) the actinomycete greatly decreased fungus growth in sand alone, but this inhibitory effect was almost removed by the presence of bentonite, suggesting that inhibition was here due almost wholly to antibiotic effect. But in medium D (high glucose), the actinomycete produced in sand + bentonite a degree of inhibition nearly half that shown in sand alone. Thus in a strongly growing culture of *Streptomyces albidoflavus* only part of the inhibition could be attributed to antibiotic action. This suggests that the inhibition of the fungus was partly due to competition by the actinomycete for some limiting nutrient and that this competition became important only when there was sufficient growth to use up the supply of this nutrient. The occurrence of very slight inhibitory effects in sand or sand + bentonite mixture moistened with media B and C was confirmed by a repeat experiment in which estimates were made on duplicate flasks.

In the experiments with media containing only low concentrations of glucose, *Streptomyces albidoflavus* had grown for 5 days before inoculation with *Fusarium culmorum*. Estimates of fungus growth were made shortly after this inoculation in an attempt to record any inhibitory effects due to the previous growth of the actinomycete. In another experiment the course of the competition was followed for a longer period of incubation and the effects of simultaneous inoculation with both organisms were observed. In order to do this it was necessary to add small quantities of glucose to the cultures at intervals in order to compensate for its removal by the growing organisms.

Twenty-four culture flasks were prepared: twelve flasks each contained 10 g. sand, and twelve flasks, 10 g. sand + bentonite mixture (1% bentonite). After sterilization the flasks were divided into two sets, each consisting of six sand flasks and six sand + bentonite flasks. Each flask received an addition of medium as follows: set 1—1 ml. ASP solution containing 10 g. glucose/l.; set 2—1 ml. ASP solution + 0.1 ml. glucose solution (1 g. glucose/l.). Each flask in set 2 also received a further addition of 0.1 ml. of glucose solution (1 g./l.) after 3, 6, 8, 12 and 16 days of incubation. Control cultures were inoculated with *Fusarium culmorum* alone, and mixed cultures were set up by two methods. In the first method 9.9×10^3 *Streptomyces albidoflavus* spores/flask were supplied at the start and incubated for 5 days at 25° before *Fusarium culmorum* was introduced at the rate of 4.39×10^4 spores/flask (series B). In the second method (series A) the two inocula were supplied at the same time (Table 4). To compensate for evaporation loss, 0.5 ml. sterile water was added to each flask weekly. Observations were made on duplicate flasks at intervals and direct count estimates of fungus growth made on one flask of each pair.

When the actinomycete was given a 5-day start (series B), it inhibited fungus growth in sand alone at both glucose concentrations, but inhibition was most marked (by comparison with the corresponding control cultures of series A) at the higher glucose concentration. At the lower glucose concentration, the presence of bentonite had no effect by 7 days in decreasing inhibition, but later, growth of the fungus recovered to about half that produced without the actinomycete and so growth was maintained. At the higher glucose concentration, the actinomycete kept down the fungal growth even in the presence of

Table 4. *Growth of Fusarium culmorum and Streptomyces albidoflavus in sand (S) and sand + bentonite (S + B) at two concentrations of glucose*

Inoculation	Glucose (%)	Type of culture	Flask no.	No. fungal spores and hyphal fragments ($\times 10^3$)/g. dry wt. culture					
				Period of incubation (days)					
				7		14		21	
Series A. Fungus alone	0.01	S	1	Mycelium	Spores	Mycelium	Spores	Mycelium	Spores
				59.9	0	123.9	1.0	—	—
	1.0	S + B	2	47.7	0	39.9	0.8	67.3	3.7
				323.3	59.8	325.3	82.1	210.6	103.0
Series B. Actinomycete, then fungus after 5 days	0.01	S	3	350.8	95.1	313.2	98.9	354.4	231.6
				11.4*	0	24.0*†	2.9	0*†	0
	1.0	S + B	4	6.3*	0	20.4*	1.2	37.6*	4.1
				0.5†	0	0†	0	0†	0
Series C. Actinomycete and fungus simultaneously	0.01	S	5	3.8†	0	15.9†	0	36.6†	10.3
				35.3*	0	43.0*†	5.1	26.9*†	0.6
	1.0	S + B	6	26.3*	0	28.2*	0.7	38.3*	3.2
				302.5	68.6	264.1*†	106.6	10.1*†	0
				327.8	143.5	285.8*	151.1	314.4*	244.1

* Slight actinomycete growth.
 † Luxuriant actinomycete growth.

† Direct actinomycete attack on fungus mycelium.
 S = sand; S + B = sand + bentonite.

bentonite, the number of mycelial fragments never exceeding 10.33 % of those in the cultures without the actinomycete.

When both organisms were inoculated simultaneously (series C), the fungus outgrew the actinomycete at first in the sand and in the sand + bentonite cultures at the higher glucose concentration (flasks 11 and 12) so no effects of the antibiotic were discerned. Later the actinomycete developed on the fungus mycelium and destroyed it (or at least its contents) in the sand cultures (cf. flask 7). This attack did not develop when bentonite was present, although the actinomycete was as abundant as in the sand cultures (flask 11). At the lower glucose concentration the fungus did not outgrow the actinomycete at the start and the fungus was suppressed by comparison with the control cultures (flasks 1 and 2), though to a lesser extent than in those cultures where the actinomycete had grown first (flasks 5 and 6). Again, bentonite had little effect on the degree of inhibition of the fungus but it did prevent the direct attack by the actinomycete which was beginning by 14 days in the sand cultures (flask 9).

Chemical tests made at the third sampling time showed some reducing sugar in all the flasks. In the cultures containing the higher concentration of glucose no nitrate or nitrite was detected, but all low concentration glucose cultures gave a strong positive test for nitrite, but no nitrate was found. In this experiment the number of actinomycete spores added to each test flask was small compared with the number of fungus spores added. This small inoculum of actinomycete did not prevent its rapid and luxuriant development, with consequent severe inhibition of fungus growth when the actinomycete was allowed to grow for 5 days before fungus inoculation in cultures moistened with medium containing 10 g. glucose/l. (flasks 7 and 8). However, though the actinomycete spores germinated rapidly in the corresponding cultures in flasks 5 and 6 which contained medium of low glucose concentration, actinomycete growth was poor and there was negligible inhibition of fungus growth either by antibiotic action or by competition for nutrients. It was considered that these competitive effects might have been more severe if the original actinomycete inoculum had been much larger. This was found to be the case in a repeat experiment in which a larger inoculum of actinomycete spores was used in cultures set up in the same way as those of set 2 in the previous experiment. The numbers of viable fungus particles increased steadily in the test flasks, in spite of the fact that the actinomycete numbers were also increasing during the same period. These increases in viable actinomycete count were due to vegetative growth since no aerial spore-bearing mycelium was observed on the sand particles; neither were spores found by microscopic examination. Nevertheless, greater fungus development was found in the control cultures than in the test cultures, although there was no initial retardation of fungus growth in the test cultures as had been observed on previous occasions when actinomycete development had been relatively much greater. The limitation of fungus growth in the test flasks was greater than had been found in similar flasks of the previous experiment, and it was considered that this limitation could be explained by a scarcity of nutrients caused by the growth of the

actinomycete. This effect had not occurred in the previous experiment when the actinomycete inoculum was much smaller.

Competition effects in sterilized soil

Two sets of culture flasks were prepared: those of one set each contained 10 g. air-dried Broadbalk (Rothamsted) soil in aggregates of 1–2 mm. diameter. Each flask of the other set contained 10 g. of similar sized aggregates of soil + bentonite mixture prepared by mixing 1% (w/w) bentonite intimately with powdered soil, mixing the whole with just sufficient water to make a stiff paste, drying and sifting. Incorporation of this amount of bentonite had no appreciable effect on the soil pH value. Mixed culture flasks of each set each received 1 ml. aqueous suspension of *Streptomyces albidoflavus* spores and each control flask 1 ml. sterile water. This volume of water was insufficient to wet all the soil but was the optimum required to ensure efficient distribution of the spores among the soil crumbs. After thorough mixing of the wet inoculated crumbs with the dry crumbs (by tapping the flasks on a rubber pad) 1.5 ml. water was added to each flask to bring the moisture content up to a value favourable for actinomycete and fungus growth. After 5 days of incubation each flask was inoculated with 1 ml. suspension of *Fusarium culmorum* spores distributed as drops over the undisturbed soil surface since the soil was then too wet to mix by agitation. Estimates of growth of the organisms were made at intervals by plate counts. Two flasks were used to make plate count estimates of fungus and actinomycete growth and observations were made on the third flask; whole flasks were used to make the suspension for plate counting. Actinomycetes were estimated by plating on starch-Tryptone agar (pH 7.0) and fungi by plating on potato-glucose agar (pH 4.5), a medium on which the actinomycete did not grow.

Some inhibition of fungus growth occurred in all the mixed cultures (Table 5). There was no reasonable doubt that the 100 mg. bentonite present

Table 5. *Growth of Fusarium culmorum and Streptomyces albidoflavus in sterilized soil*

		Initial inoculum (no. viable particles)	Period of incubation after inoculation with fungus (days)			
			0	6	13	36
		No. viable fungus particles ($\times 10^3$)/flask				
Soil	A + F	15.3	—	12.1	22.5	62.6
	F	15.3	—	912.5	4000	4331
Soil + bentonite	A + F	15.3	—	10.2	30.6	68.3
	F	15.3	—	793.8	3363	3263
		No. viable actinomycete particles ($\times 10^6$)/flask				
Soil		0.1	84.9	370.4	412.8	451.3
Soil + bentonite		0.1	75.4	237.1	308.1	417.2

F = fungus alone; A + F = actinomycete + fungus.

in each soil + bentonite culture inactivated any antibiotic formed by the actinomycete from nutrients naturally present in the soil; consequently, suppression of fungus development in these mixed cultures was attributed to competition between the organisms for nutrients or for space. However, since the numbers of fungus particles in the mixed cultures in soil + bentonite were at all times comparable with those in the mixed cultures in soil, it was concluded that the conditions in both kinds of cultures were similar, and that the amount of soil present (10 g.) was itself adequate to inactivate antibiotic produced by the actinomycete.

The severity of inhibition of fungus growth in this experiment may, perhaps, be explained by rapid exhaustion of nutrients in the surface layers of the soil crumbs by the actinomycete even though the deeper parts might still perhaps contain a reserve of nutrient materials. In neither type of test culture was there evidence that the actinomycete had attacked the fungus directly. This direct attack was further investigated.

The existence of a lytic actinomycete secretion was demonstrated on solid media. Eight different agar media were prepared with and without added clays (Table 6). One Petri dish of each medium was inoculated with *Fusarium culmorum* and incubated. After 13 days, three standardized colonies of *Streptomyces albidoflavus* (Skinner, 1950) were prepared on each plate and the incubation continued.

Table 6. *Direct actinomycete attack on mycelium of Fusarium culmorum on AS solution agar media*

K ₂ HPO ₄ concentration ...	0.005 g./l.				0.5 g./l.			
	0.1 g./l.		10 g./l.		0.1 g./l.		10 g./l.	
	5	40	5	40	5	40	5	40
Glucose concentration ...								
C:N ratio (nitrate concentration adjusted) ...								
A. No clay	—	—	+	+	—	—	—	—
			5.3	4.8				
B. Agar medium + bentonite	—	—	+	—	—	—	—	—
			1.25					
C. Agar medium + Broadbalk clay	—	—	+	+	—	—	—	—
			1.0	0.3				

— = no attack on the fungus; + = direct attack on fungus mycelium as shown by loss of staining properties near actinomycete colonies. Figures give mean width (mm.) of non-staining zones.

The plates were kept under observation for 3 weeks; during this time no attack on the fungus was seen. However, when each plate was flooded with acetic acid-aniline blue stain, allowed to stand for 1 hr. and then washed with water, it was obvious that attack had developed but only on media containing 10 g. glucose/l. and very little phosphate. In no case did the actinomycete grow directly on the fungus mycelium but each actinomycete colony was surrounded by a clear zone in which the fungus hyphae did not take up the stain. These unstained hyphae were apparently devoid of contents although the

hyphal walls were intact. This partial lysis was prevented or very much reduced (as judged by the widths of the clear zones) when clay was present.

Direct attack by *Streptomyces albidoflavus* on hyphae of *Fusarium culmorum*, as had been noticed in sand culture, also took place in water. Washed minute discrete colonies of *F. culmorum* (prepared by growing in glucose asparagine solution agitated on a slow-speed shaker for 40 hr.) were suspended in distilled water and incubated with an inoculum of *Streptomyces albidoflavus*. The actinomycete developed luxuriantly on the fungus mycelium during the first 10 days. After about 3 weeks very little fungus mycelium capable of taking up acetic acid-aniline blue was found though it appeared that the walls of this mycelium had not been attacked. In similar cultures containing bentonite (1 mg. or 10 mg./l. culture) the actinomycete was less intimately associated with the fungus and the staining properties of the latter were almost unimpaired. Probably the same agent (e.g. an enzyme readily adsorbed by bentonite and other clays) was responsible both for the partial lysis on agar media and for the direct attack on the fungus in aqueous suspension.

The possibility that the lytic agent was also the antibiotic was then investigated. One ml. portions of a suspension of minute fungus colonies, prepared as described above, were added to 5 ml. portions of: (a) sterile water; (b) filtrates of liquid *Streptomyces albidoflavus* cultures (glucose asparagine solution); (c) heated culture filtrates. Duplicate tubes were incubated and samples removed at intervals (up to 3 weeks) for microscopic examination. In the water tubes the contents of the fungus hyphae were almost entirely stainable with acetic acid-aniline blue though some parts of the hyphae had lost their ability to take up this stain. The condition of the fungus in the unheated and heated culture filtrates was essentially the same as in water. Thus there was no evidence that any lytic substance was present in the filtrates, even though they contained sufficient antibiotic to prevent any new growth of the fungus. It seemed, therefore, from these results, and from those of the experiment in which strong direct attack had taken place on the fungus in water (i.e. under conditions of nutrition unlikely to favour antibiotic production), that the antibiotic and the lytic agent were different substances.

DISCUSSION

In sand culture the addition of bentonite and other clays decreased but did not prevent the inhibition of growth of *Fusarium culmorum* by *Streptomyces albidoflavus*. This indicated that the inhibitory effect of the actinomycete in sand was only in part due to antibiotic action, since bentonite inactivated the antibiotic in the amounts used. Inhibition in the presence of bentonite may reasonably be attributed to competition between the organisms for limiting nutrients. However, it cannot be asserted that competition for nutrients was as important in sand cultures, where the antibiotic was already suppressing growth of the fungus to an extent depending on nutrient conditions, as it was in the sand + bentonite cultures where antibiotic action had undoubtedly been checked by the clay. A lysis of the contents of fungus

hyphae by actinomycete mycelium in contact with it was observed in sand culture. This lytic attack which could also be demonstrated in aqueous suspensions of the fungus and on agar media, was always prevented or greatly reduced by bentonite which, presumably, adsorbed the lytic agent.

In sand cultures there was a direct relation between the amount of glucose supplied and the antagonistic effects of the actinomycete (Skinner, 1956). This relation was also found when bentonite was added to the sand, so a high concentration of glucose presumably increased competition for limiting nutrients as well as influencing the production of antibiotic secretions. Rapid growth of one organism because of the presence of abundant glucose probably leads to exhaustion of one or more of the other nutrients (e.g. N source, phosphate) and in this way limits development of the other organisms. This exhaustion did not apparently take place when glucose was present in low concentration. In this sense, competition for nutrients is likely to be more severe when energy-yielding materials are abundant than when they are relatively scarce.

Lysis of the fungus mycelium was also most evident at high concentrations of glucose. The experiment in which fungus mycelium suspended in water was attacked readily by the actinomycete suggests that production of the lytic agent was not directly dependent on the presence of a high concentration of glucose but was determined primarily by the presence of the mycelium itself. However, production of mycelium is favoured by adequate supplies of glucose. At low concentrations of glucose, even when maintained by successive additions, very little inhibition of the fungus by the actinomycete (due to antibiotic activity, lytic action or competition for nutrients) or suppression of the actinomycete by the fungus (due presumably to rapid exhaustion of nutrients by the faster-growing fungus) was found even in sand without bentonite; both organisms were then able to survive together, though the growth of both was comparatively poor.

There is evidence (Skinner, Jones & Mollison, 1952) that micro-organisms exist mainly as very small colonies in soil, and that the luxuriant growth of any one viable unit (e.g. a spore, vegetative cell or a fragment of mycelium) may be comparatively rare. Soil conditions seem to approximate to those in the experimental cultures in which nutrient concentrations are low, i.e. when both organisms are able to grow together slowly under nutrient conditions unfavourable to the rapid and luxuriant growth of either. It may well be that in soil so many different organisms are able to co-exist because the nutrient conditions are generally unfavourable to the rapid growth of any one of them, i.e. growth is suppressed below the degree at which nutrients are rapidly exhausted and at which production of antibiotics, and lysis, etc., can become effective. The failure to demonstrate antibiotic action in sterile soil does not preclude the possibility that *Streptomyces albidoflavus* may sometimes be able, in certain soils, to produce sufficient antibiotic to affect the growth of *Fusarium culmorum* or other fungi. This consideration applies with equal force to other actinomycetes, many of which produce antibiotics.

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The Action of Isonicotinic Acid Hydrazide on the Metabolism of *Mycobacterium smegmatis*

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SUMMARY: The effect of various substrates on the oxygen uptake of *Mycobacterium smegmatis* grown in the presence of glucose and glycerol was investigated; isoniazid had no effect on these oxidations. Three different effects of isoniazid on the metabolism of this organism were demonstrated. (i) Growth of the organism in a minimal medium in the Warburg apparatus was prevented by the addition of isoniazid, the amount required being related to the number of organisms present, their age and their sensitivity to the drug as measured by serial dilution in the test tube. (ii) In confirmation of the report of Zeller, Barsky, Berman & Fouts (1952) it was found that isoniazid inhibited the oxidation of putrescine by *M. smegmatis*. (iii) Following the work of Gray (1953) the effect of isoniazid on the oxidation of various substrates by *M. smegmatis* grown in the absence of glucose and glycerol was studied. Isoniazid caused a 30% inhibition of the oxidation of acetate. The amount of isoniazid required to inhibit the oxidation of putrescine and acetate was the same whether the strain used was sensitive or resistant to isoniazid as measured by growth in the test tube.

In an attempt to discover the mechanism by which isonicotinic acid hydrazide (isoniazid) inhibits the growth of mycobacteria, the effect of isoniazid on their respiration was investigated. Preliminary work involved a study of the oxidative metabolism of the saprophytic organism *Mycobacterium smegmatis*.

METHODS

Organisms. The parent strain of *Mycobacterium smegmatis* was obtained from the National Collection of Type Cultures (7011). It was maintained on nutrient agar and subcultivated into Dubos fluid medium 48 hr. before use. This parent strain, *M. smegmatis* S, was inhibited by 8 µg. isoniazid/ml. By serial transfer from the highest concentration of isoniazid in which growth occurred, into higher concentrations of the drug, strains were obtained which were resistant to 200 µg. isoniazid/ml. (*M. smegmatis* R₁) and to 400 µg. isoniazid/ml. (*M. smegmatis* R₂).

Media

Dubos fluid medium (Dubos & Davis, 1946). This was supplied by the Southern Group Laboratory, Park Hospital, Hither Green, London, S.E. 13. The basal medium containing Tween 80 was supplied in 100 ml. amounts, and a 9% (w/v) solution of bovine albumin fraction V (Armour) in water in 5 ml. amounts. The bovine albumin from several bottles was pooled, and reesterilized by filtration through a Seitz E.K. filter. For use, 4 ml. of this albumin solution was added to each 100 ml. of Dubos medium.

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Dubos & Middlebrook medium for heavy yield of organisms (Dubos & Middlebrook, 1947). This was prepared by adding magnesium sulphate, sodium citrate, glycerol, and glucose to the Dubos medium as used above.

Fumarate medium. This was a modification of the Dubos medium, and contained, in 1 l. of medium: KH_2PO_4 , 1 g.; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 6.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g.; casein hydrolysate (20 %, w/v), 10 ml.; Tween 80, 10 % (v/v), 5 ml.; sodium fumarate 1.7 g. The pH value was adjusted to 6.8 and the medium sterilized by autoclaving in 100 ml. amounts at 10 lb. for 10 min.

Sensitivity tests

These were carried out in test tubes ($6 \times \frac{5}{8}$ in.), each containing 4 ml. of Dubos fluid medium. The inoculum was 1 drop (0.02 ml.) of a 48 hr. culture in Dubos medium. Incubation was at 37°.

Warburg experiments

The organisms were grown in Roux bottles, each containing 100 ml. of medium, and incubated flat for 3 days before harvesting. The cultures were collected by centrifugation at 3000 r.p.m. for 15 min., washed by resuspending in 0.2M-phosphate buffer (pH 6.8) and centrifuged again at 3000 r.p.m. for a further 15 min. For use the organisms were resuspended in phosphate buffer, and the suspensions standardized in the Unicam absorptiometer. One ml. of this suspension was used in each Warburg vessel which had a total fluid volume of 3.2 ml. All substances to be tested were dissolved so that the desired amount was contained in 0.6 ml. distilled water and added from the side arm; 0.2 ml. 20 % KOH was used to absorb CO_2 . The temperature of the bath was 37° and the shaking rate 100 strokes/min. Results are expressed in terms of Q_{O_2} , i.e. $\mu\text{l. O}_2$ taken up/mg. dry wt. organisms/hr.

RESULTS

Oxidative reactions of organisms grown in Dubos & Middlebrook medium

In order to obtain a high yield of organisms and so counteract some of the experimental difficulties of using mycobacteria, the organisms were grown in Dubos & Middlebrook medium. This contains salts, glycerol, casein hydrolysate and glucose, and a good yield was obtained after 3 days of incubation.

Effect of isoniazid on the oxidation of single substrates. The effect of various single substrates upon the oxygen uptake of washed cell suspensions of *Mycobacterium smegmatis* was investigated. The substances tested included glycerol, glucose, components of the Krebs citric acid cycle, several amino acids, and certain benzoic acid derivatives. The results obtained and the effect of isoniazid on these oxidations are shown in Table 1. Although the concentration of isoniazid used (10^{-1}M) was far greater than that required to inhibit growth in the test tube ($5 \times 10^{-4}\text{M}$), the addition of isoniazid caused no change in the Q_{O_2} under the conditions used, in the presence of any of the substrates tested.

In view of the work of Umbreit (1953) on the effect of streptomycin on the reaction between pyruvate and oxaloacetate, the effect of isoniazid on oxygen uptake from oxaloacetate in the presence of pyruvate was tested. This also was unchanged by the addition of isoniazid.

Table 1. *Effect of isoniazid on oxygen uptake of Mycobacterium smegmatis*

Substrate (conc. 10^{-2} M)	Q_{O_2}	Q_{O_2} in presence of (0.1 M) isoniazid
No addition	13.6	13.6
Asparagine	39.4	39.2
Sodium acetate	49.8	49.8
α -Alanine	38.6	38.6
β -Alanine	40	40.5
Sodium benzoate	37.8	38.1
<i>p</i> -Aminobenzoic acid	13.0	13.2
<i>p</i> -Hydroxybenzoic acid	13.5	13.8
Anthranilic acid	12.9	12.8
Glutamic acid	28.2	28.0
Glyceraldehyde	26.8	26.8
Glycerol	22.4	22.5
Glucose	29.6	30.1
L-Histidine HCl	26.3	27
Sodium lactate	29.4	30.2
Sodium citrate	13.6	13.2
L-Leucine	29.1	28.5
Sodium oxaloacetate	26.4	27.1
Sodium pyruvate	34	33.4
Serine	30	30
Sodium succinate	31	29.6
Tyrosine	20.4	20
Tryptophan	29.4	29.2
Oxaloacetate + pyruvate	60.8	60.3

Effect of isoniazid on the oxygen uptake of 'growing' organisms. Since no inhibition of oxidative reactions by isoniazid was found with resting suspensions of *Mycobacterium smegmatis*, it was decided to study the effect of isoniazid on oxygen uptake during growth in the Warburg apparatus. A suspension of *M. smegmatis* already growing at 37° in Dubos & Middlebrook medium was transferred directly, without washing, to Warburg flasks. In the presence of isoniazid (10^{-4} M) the Q_{O_2} was steady over a period of 7 hr., but in its absence the Q_{O_2} increased at a constant rate (Fig. 1). That this increase in the rate of oxygen uptake in the absence of isoniazid was due to growth, is suggested by the following points:

(1) At the end of 7 hr. there was an increase in the mass of organisms. This was visible to the naked eye and was also shown by measuring the turbidity in the Unicam absorptiometer.

(2) By plotting the oxygen uptake against the square of time, a linear relation was shown (Fig. 1). This arithmetic type of growth pattern was found by Fisher & Kirchheimer (1952) to be typical of the growth of mycobacteria under certain conditions. Later Halpern & Kirchheimer (1954) showed that the growth reverted to the more usual logarithmic type of growth on shaking.

(3) Organisms grown for 24 hr. before transferring to Warburg flasks were more active than those grown for 48 hr. or longer.

(4) More dilute suspensions of organisms showed a more rapid increase in rate of oxygen uptake than heavier ones.

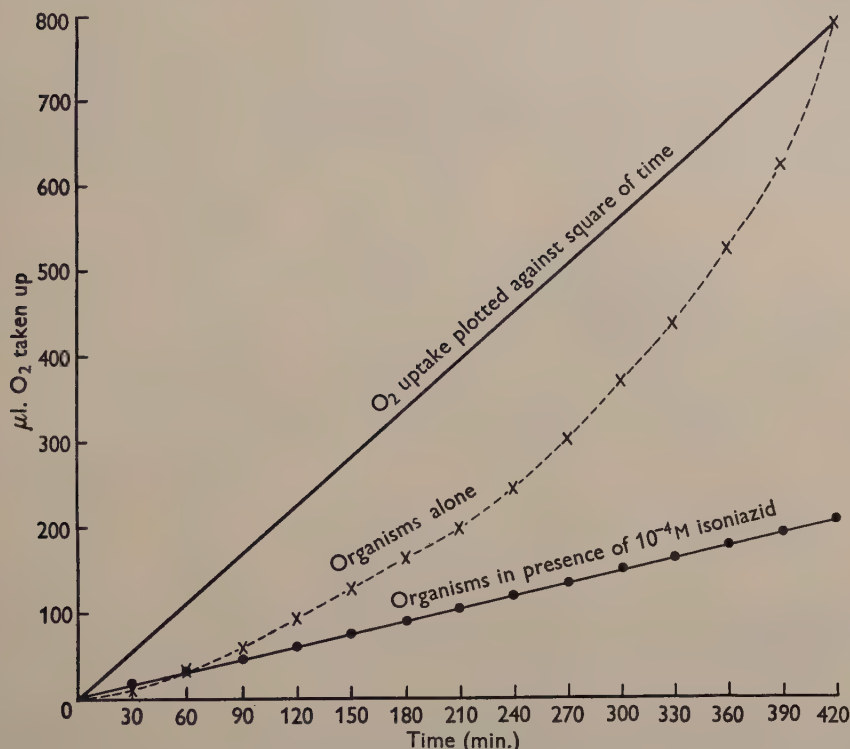


Fig. 1. Growth of *Mycobacterium smegmatis* in the Warburg apparatus and its inhibition by isoniazid.

Attempts were made to simplify this system which showed isoniazid inhibition. An increase in Q_{O_2} was also obtained after washing the organisms twice with phosphate buffer, and resuspending them in 0.2M-phosphate (pH 6.8) containing 0.2% (w/v) casein hydrolysate and 0.1M-glycerol. The rate of increase of Q_{O_2} in this simplified system was unaffected by the addition of albumin, Tween 80, magnesium sulphate, sodium citrate, glucose, tuberculin purified protein derivative (P.P.D.), phthiocol, *p*-aminobenzoic acid or pyridoxin.

When the casein hydrolysate in the simplified system was replaced by putrescine, aspartic acid, asparagine, serine, ammonium sulphate or ammonium citrate the 'growth' effect was not observed. The glycerol could not be replaced by glyceraldehyde or glucose. The concentration of isoniazid required to inhibit 'growth' was related to that required to inhibit growth in Dubos fluid medium in the test tube (Table 2).

Antagonists. Several substances were tested as antagonists of isoniazid inhibition in this system. It was thought possible that inhibition of growth by isoniazid might be due to its competitive antagonism with pyridoxin or nicotinic acid, because of similarity of structure. Pyridoxin (10^{-2} to 10^{-5} M), pyridoxal (5×10^{-5} to 10^{-7} M) and nicotinic acid (10^{-2} to 10^{-5} M) had no effect on the system; they did not change the rate of increase of oxygen uptake nor

Table 2. *Relation between amounts of isoniazid required to inhibit growth of strains of Mycobacterium smegmatis in test tubes and in Warburg flasks*

Organism	Isoniazid required for inhibition	
	In test tube ($\mu\text{g./ml.}$)	In Warburg flasks ($\mu\text{g./ml.}$)
<i>M. smegmatis</i> S	6	10
<i>M. smegmatis</i> R ₁	200	200
<i>M. smegmatis</i> R ₂	400	420

Table 3. *Effect of isoniazid on acetate oxidation by Mycobacterium smegmatis grown in the absence of glucose or glycerol*

Strain	Isoniazid (M)	Q_{O_2} (basal)	Q_{O_2} in presence of acetate (0.1 M)	Inhibition (%)
<i>M. smegmatis</i> S	0	1.3	13	—
	10^{-2} M	1.3	9.5	31
	10^{-3} M	1.3	13.1	0
<i>M. smegmatis</i> R ₁	0	1.32	13.0	—
	10^{-2} M	1.31	9.4	31
	10^{-3} M	1.33	13.0	0

the action of isoniazid in suppressing this. Manganese chloride (10^{-3} to 10^{-4} M), sodium citrate (10^{-2} to 10^{-4} M) and haemin (10^{-3} to 10^{-6} M) were used, following reports by Fisher (1954) that they antagonized the inhibition by isoniazid of growth of *Mycobacterium tuberculosis* H37 Rv. The screening plate technique used as described by King, Knox & Woodroffe (1953), indicated that salicylic acid hydrazide, nicotinic acid hydrazide and benzhydrazide, antagonized the inhibitory action of isoniazid on *M. smegmatis* in growth (Anderson, King, Knox & Meadow, 1953). These substances were also tested at concentrations of 10^{-2} to 10^{-4} M in the above system but were without effect.

Oxidative reactions of cells grown in the absence of glucose and glycerol

Gray (1953) showed that inhibition by isoniazid of oxygen uptake by mycobacteria occurred only when the organisms were grown in a medium free from glucose and glycerol, or were starved in aerated phosphate buffer after growth. The effect of isoniazid on the oxidation of single substrates by *Mycobacterium smegmatis* grown in the absence of glucose and glycerol was therefore tested. The organisms were grown for 3 days in the fumarate medium; they had a much lower basal Q_{O_2} than the organisms used previously. Table 3 shows the

results obtained when isoniazid-sensitive and isoniazid-resistant strains of *M. smegmatis* were used with acetate as substrate. 10^{-2} M-isoniazid caused a 30 % inhibition of the oxygen uptake from acetate whether or not the strain used was sensitive to isoniazid as measured by growth in Dubos medium in the test tube.

Diamine oxidase

One of the earlier reports of enzyme inhibition by isoniazid was that of Zeller *et al.* (1952) who described the effect of isoniazid on the diamine oxidase of cell suspensions and soluble enzyme preparations of *Mycobacterium smegmatis*. The most active of the diamine oxidases in this organism is the putrescine

Table 4. *Action of isoniazid on putrescine oxidase of Mycobacterium smegmatis*

The organisms were grown in Dubos & Middlebrook medium containing 0.5 % (w/v) putrescine.

Putrescine concentration (M)	Isoniazid concentration (M)	Q _{O₂}	Inhibition (%)
0	0	4.7	—
0.01	0	12.1	—
0.01	10^{-2}	6.5	72
0.01	10^{-3}	6.45	72
0.01	10^{-4}	12.0	0

oxidase, and this was tested in the Warburg apparatus and the effect of adding isoniazid studied. In confirmation of the report of Zeller *et al.* (1952) it was found that putrescine was oxidized by washed suspensions of *M. smegmatis* and that isoniazid at 10^{-3} M caused a 72 % inhibition of this oxidation in both sensitive and resistant strains (Table 4).

Arginine decarboxylase

Yoneda & Asano (1953) reported that the arginine decarboxylase of a strain of *Escherichia coli* was inhibited by high concentrations of isoniazid, the inhibition being reversed by pyridoxin and more effectively by high concentrations of pyridoxal. This work was repeated with *Escherichia coli communius* (NCTC 6064) grown for 16 hr. at 25° in 2 % (w/v) glucose broth to which had been added 0.001 % (w/v) arginine. The arginine decarboxylase was measured by the evolution of carbon dioxide after the addition of arginine (to 3×10^{-3} M) to washed suspensions in McIlvaine's citric acid buffer (pH 4.2). As shown in Table 5, isoniazid inhibited the action of the arginine decarboxylase, and this inhibition was partially reversed by pyridoxin (10^{-2} M). Attempts were made to demonstrate a similar effect with washed suspensions of *Mycobacterium smegmatis*. The organisms were grown in broth containing 2 % (w/v) glucose and 0.001 % (w/v) arginine at 25 and at 37°, and also in Dubos & Middlebrook medium to which 0.001 % (w/v) arginine had been added. No arginine decarboxylase was found in any of these organisms.

Table 5. *The effect of isoniazid and pyridoxin on the arginine decarboxylase of Escherichia coli communius*

Isoniazid concentration (M)	Pyridoxin concentration (M)	Q_{O_2}	Inhibition (%)	Annulment of inhibition (%)
0	0	60	—	—
10^{-2}	0	7	88	—
10^{-3}	0	48	20	—
10^{-4}	0	68	0	—
0	10^{-2}	62	0	—
10^{-2}	10^{-2}	24	—	32

DISCUSSION

The systems so far shown to be inhibited by isoniazid are the putrescine oxidase and the acetate oxidase of *Mycobacterium smegmatis*, 'growth' of *M. smegmatis* in a minimal medium and the arginine decarboxylase of *Escherichia coli*. It appears unlikely that inhibition of the putrescine oxidase of *Mycobacterium smegmatis* is the only, or indeed the major, site of action of isoniazid for two main reasons: (1) Inhibition of diamine oxidases is a general reaction given by many substances (including hydroxylamine, nicotinic acid hydrazide and streptomycin), and it does not appear to be related to their antituberculous activity (Zeller, Owen & Karlson, 1951). (2) Certain diamines, notably spermine, are inhibitory to the growth of tubercle bacilli only in the presence of the diamine oxidase (Hirsch & Dubos, 1952). If isoniazid acts only as an inhibitor of the diamine oxidase, it should therefore permit growth, rather than inhibit it, in the presence of diamines. No such isoniazid dependence has been demonstrable in growth experiments in the presence of diamines. The presence of an arginine decarboxylase in *M. smegmatis* has not been demonstrated. It seems unlikely, therefore, that inhibition of arginine decarboxylase by isoniazid plays a part in its inhibition of the growth of mycobacteria.

The chief effect of isoniazid on 'growth' as followed in the Warburg apparatus seems to resemble fairly closely conditions in the test tube, in which growth inhibition is known to occur, since the amounts of isoniazid required for inhibition in the two systems are comparable. This is not, however, an easy technique with which to work since the low concentrations of organisms required for optimum 'growth' necessitate the use of organisms which are not clumped together. Mechanical grinding, however gentle, prevents the 'growth' effect, and it is therefore difficult to be sure of obtaining reasonably uniform distributions of the organisms into each Warburg flask. It was thought probably not profitable to continue using the technique as a means of demonstrating the activity of isoniazid, particularly in view of the fact that no such effect was demonstrable with *Mycobacterium tuberculosis* var. *bovis* (B.C.G.).

The most hopeful line of approach to the problem appears to be the inhibition of acetate oxidation by isoniazid, using organisms grown in a medium free from glucose and glycerol. However, the use of *Mycobacterium smegmatis* in

this work is limited by the fact that its sensitivity to isoniazid as measured by this technique is not related to its sensitivity to the drug in growth tests, and that it is considerably less sensitive to isoniazid than other members of the genus *Mycobacterium* such as *M. tuberculosis* var. *hominis* (H37 Rv and H37 Ra) and *M. tuberculosis* var. *bovis* (B.C.G.).

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The Effect of Isonicotinic Acid Hydrazide on the Oxidative Metabolism of *Mycobacterium tuberculosis* var. *bovis* B.C.G.

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SUMMARY: Isoniazid inhibited the oxidation of acetate in the Warburg apparatus by *Mycobacterium tuberculosis* B.C.G. and other mycobacteria but not by any of the other organisms tested. This effect was investigated in more detail with B.C.G. Inhibition of acetate oxidation was obtained by isoniazid whatever medium had been used for growth. The amount of isoniazid required for inhibition was related to the number of organisms present and their sensitivity to isoniazid as measured by the usual test-tube method. None of the substances reported to antagonize isoniazid inhibition was effective in annulling inhibition by isoniazid in this acetate oxidation system. The effect of various mixtures of drugs on acetate oxidation was tested. Concentrations of isoniazid and streptomycin which when used singly were insufficient to inhibit acetate oxidation, were inhibitory when used together. Similarly, mixtures of isoniazid + *p*-aminosalicylic acid (P.A.S.) or terramycin, or of streptomycin + P.A.S., at concentrations which were subinhibitory when used singly, were also effective in inhibiting acetate oxidation. It is suggested that the action of drugs or drug mixtures could usefully be investigated by this or similar techniques which have the advantage of largely eliminating the selection of resistant strains.

In a previous communication (Meadow, 1956) the effect of isonicotinic acid hydrazide (isoniazid) in inhibiting the oxidative metabolism of *Mycobacterium smegmatis* was described. To show any such inhibition, however, it was necessary to use high concentrations of isoniazid and carefully defined experimental conditions. Further experiments were therefore undertaken with an organism initially about 100 times more sensitive to isoniazid than *M. smegmatis*. The organism used was the attenuated strain *M. tuberculosis* var. *bovis* B.C.G.

METHODS

Organisms used

Mycobacterium tuberculosis var. *bovis* B.C.G. This was originally obtained from Dr R. J. W. Rees (National Institute for Medical Research, Mill Hill, London, N.W. 7). The parent strain was inhibited by 0.07 μ g. isoniazid/ml.; and an isoniazid-resistant variant, obtained by serial transfer from the highest concentration of isoniazid in which growth occurred into higher concentrations, grew in 5-10 μ g. isoniazid/ml. The strains were maintained in Dubos fluid medium, and subcultured at fortnightly intervals.

Mycobacterium smegmatis NCTC 523. The strain was maintained on nutrient agar and subcultured into Dubos fluid medium 24 hr. before use. It was inhibited by 8 μ g. isoniazid/ml.

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Mycobacterium tuberculosis var. *hominis* H37 Ra. This was originally obtained from Dr R. J. W. Rees; it was sensitive to 0.1 µg. isoniazid/ml. and was maintained in Dubos fluid medium.

Staphylococcus aureus Oxford NCTC 6571; *Escherichia coli communius* NCTC 6064; *Bacillus cereus* NRRL 569.

Media

The Dubos, Dubos & Middlebrook, and fumarate media used were described previously (Meadow, 1956). Succinate medium. This was prepared as for the fumarate medium but with the sodium fumarate replaced by 0.65 g. succinic acid. Tarshis medium (Tarshis, Kinsella & Parker, 1958) was an agar medium containing blood, glycerol and penicillin.

Sensitivity tests

These were carried out in test tubes ($6 \times \frac{5}{8}$ in.), each containing 4 ml. of Dubos fluid medium. The inoculum was 1 drop (0.02 ml.) of a 10-day culture in Dubos medium. Incubation was at 37°.

Warburg experiments

The cells were grown in Roux bottles, each containing 100 ml. of medium and incubated flat for 14 days at 37° before reaping. The preparation and use of the cells in the Warburg apparatus have been described in a previous communication (Meadow, 1956).

RESULTS

The work described previously (Meadow, 1956) had indicated that a suitable means for studying inhibition of oxidative metabolism by isoniazid might be the oxidation of acetate by organisms grown in the absence of glucose and glycerol. In view of the specificity of low concentrations of isoniazid in inhibiting the growth only of members of the genus *Mycobacterium*, any system inhibited by isoniazid should be specific to mycobacteria in this respect. A small selection of organisms was tested for their ability to oxidize acetate and the effect of isoniazid on this oxidation.

Organism specificity

The organisms tested included members of the genus *Mycobacterium*, a staphylococcus, a strain of *Bacillus cereus*, and a strain of *Escherichia coli* whose arginine decarboxylase was sensitive to isoniazid. As shown in Table 1 only mycobacteria were inhibited; B.C.G. and H37 Ra were inhibited whether grown in the presence or absence of glucose or glycerol; *Mycobacterium smegmatis* showed inhibition only when grown in the absence of glucose and glycerol. The staphylococcus, *Bacillus cereus* and *Escherichia coli* strains showed no inhibition under any of the growth conditions used.

The experiments described in the sequel relate to *Mycobacterium tuberculosis* var. *bovis* B.C.G.

Substrate specificity for B.C.G.

With washed suspensions of B.C.G. grown in a medium containing neither glucose nor glycerol, but with fumarate as sole carbon source, the effect of various substrates on their oxidative metabolism was investigated with and without the further addition of isoniazid. The substrates tested and the

Table 1. *Specificity of isoniazid inhibition of acetate oxidation for various mycobacteria*

Organism	Growth medium	Basal Q_{O_2}	Q_{O_2} and acetate $10^{-1}M$	Q_{O_2} and acetate $10^{-3}M$ + isoniazid $2 \times 10^{-1}M$	Inhibition by isoniazid (%)
<i>Mycobacterium smegmatis</i>	Dubos & Middlebrook	14.6	28.4	28.3	0
	Fumarate	4.4	8.6	6.9	41
B.C.G.	Dubos & Middlebrook	7.2	15.8	12.2	47.4
	Fumarate	1.4	5.1	3.2	50
<i>Mycobacterium tuberculosis</i> H37 Ra	Dubos & Middlebrook	7.8	17.6	13.1	49
	Fumarate	3.9	8.3	6.5	49
<i>Staphylococcus aureus</i>	Digest broth	1.2	2.61	2.6	0
	Dubos & Middlebrook	1.4	2.8	2.7	0
	Fumarate	2.2	4.6	4.5	0
<i>Escherichia coli communius</i>	Digest broth	4.0	13.0	13.1	0
	Dubos & Middlebrook	3.8	9.1	8.9	0
	Fumarate	2.4	4.9	4.9	0
<i>Bacillus cereus</i>	Digest broth	2.3	10.6	10.8	0
	Fumarate	1.1	7.4	7.2	0

Table 2. *Effect of isoniazid on the oxygen uptake of B.C.G. with various substrates*

The fumarate growth medium was used throughout. Acids listed were added as sodium salts.

Substrate added (0.01 M)	Q_{O_2}	Q_{O_2} in presence of isoniazid (0.001 M)	Inhibition by isoniazid (%)
—	2.4	2.4	0
Acetate	5.1	3.75	50
Glucose	5.1	5.2	0
Glycerol	4.9	4.8	0
Succinate	2.4	2.4	0
Malate	2.35	2.35	0
Fumarate	2.3	2.3	0
Pyruvate	3.6	3.0	50
Lactate	4.2	3.3	50
Lactose	2.4	2.4	0
Citrate	2.35	2.35	0
Formate	2.4	2.4	0

results are shown in Table 2. It may be seen that the increase of Q_{O_2} above the basal rate, caused by the addition of acetate, pyruvate or lactate to the system, was approximately halved by the addition of isoniazid.

Since the stimulation of respiration by acetate was greater than that by pyruvate or lactate, and it seemed unlikely that its inhibition by isoniazid

was due to chemical combination between the two substances, it was decided to investigate further this acetate inhibition system.

Effect of different growth media on isoniazid inhibition

In view of the different results obtained with *Mycobacterium smegmatis* depending on the medium used for growth, suspensions of B.C.G. which had been grown in different media were tested. Acetate oxidation was inhibited by isoniazid, whereas oxidation of glucose and glycerol was unchanged, under all the growth conditions tested (Table 3). Succinate was oxidized only when the organisms had been grown in the presence of succinate, this oxidation being partially inhibited by isoniazid. In further experiments organisms grown in the fumarate medium were used.

Table 3. *Effect of isoniazid on oxidative metabolism of B.C.G. grown in different media*

Growth medium	Substrate added (0.01 M)	Q_{O_2}	Q_{O_2} in presence of isoniazid (0.001 M)	Inhibition by isoniazid (%)
Dubos	—	1.98	1.99	0
	Acetate	16.1	9.3	50
	Glucose	4.2	4.3	0
	Glycerol	5.1	5.0	0
	Succinate	2.0	2.0	0
Dubos & Middlebrook	—	8.2	8.3	0
	Acetate	15.8	12.2	47.5
	Glucose	10.4	10.3	0
	Glycerol	9.8	9.9	0
	Succinate	8.3	8.2	0
Tarshis	—	8.5	8.6	0
	Acetate	26.5	16.5	45
	Glucose	17.1	17.2	0
	Glycerol	17.8	17.9	0
	Succinate	8.9	8.7	0
Succinate	—	3.8	3.85	0
	Acetate	10.4	7.1	50
	Glucose	10.1	10.3	0
	Glycerol	12.4	12.3	0
	Succinate	5.4	4.5	50

Isoniazid concentration required for inhibition of acetate oxidation

The effect of adding different concentrations of isoniazid to suspensions of B.C.G. in the presence of acetate was tested. Even pre-incubation of the organisms with high concentrations of isoniazid in phosphate buffer for 24 hr. before use produced nothing greater than a 50 % inhibition of acetate oxidation. The concentration of isoniazid required to produce this inhibition was dependent on the concentration of organisms present and on their sensitivity to isoniazid in growth.

Fig. 1 shows the effect of concentration of organisms on isoniazid inhibition; smaller amounts of isoniazid were required to produce 50 % inhibition in the more dilute suspensions.

Table 4 shows the relation between the amount of isoniazid required for 50% inhibition of acetate oxidation, and the sensitivity of B.C.G. as measured by growth in the test tube. It may be seen that the isoniazid-resistant strain required a much higher concentration of isoniazid to produce the same effect on acetate oxidation.

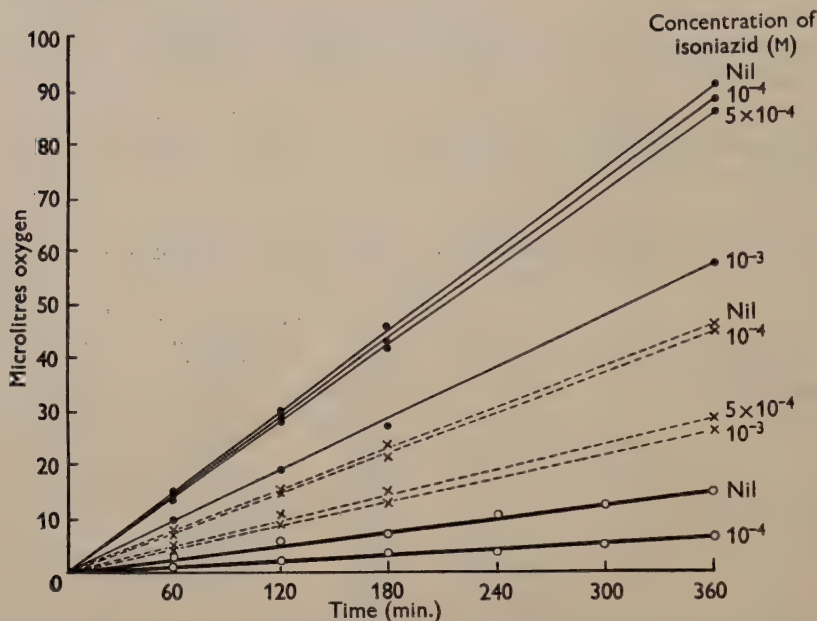


Fig. 1. Effect of cell concentration on amounts of isoniazid required to inhibit acetate oxidation by B.C.G. (S). Organisms grown in fumarate medium for 14 days, resuspended in 0.025 M-phosphate buffer pH 6.8. 0.1 M-acetate present in each flask. 0.3 ml. isoniazid added from the side arm. Gas phase; air. Temperature 37°. ●—●, 1 mg. dry wt. organism/ml.; × - - ×, 0.5 mg. dry wt./ml.; ○—○, 0.25 mg. dry wt./ml.

Table 4. Relationship of isoniazid sensitivity of B.C.G. strains to inhibition of acetate oxidation by isoniazid

Organism	Concentration of isoniazid required to inhibit growth (M)	Substrate added	Q _{O₂}	Isoniazid added to suspension in Warburg vessel (M)	Inhibition by isoniazid (%)
B.C.G. (S)	10 ⁻⁶	—	2.2	0	—
		Acetate	12.6	0	—
		Acetate	12.5	10 ⁻⁴	0
		Acetate	8.3	5 × 10 ⁻⁴	42
		Acetate	8.0	10 ⁻³	45
		Acetate	8.1	10 ⁻²	44
B.C.G. (R)	10 ⁻⁴	—	1.7	0	—
		Acetate	13.1	0	—
		Acetate	13.3	10 ⁻²	0
		Acetate	13.0	10 ⁻¹	0
		Acetate	7.7	3 × 10 ⁻¹	48

Antagonists of isoniazid inhibition

There have been several reports of substances reputed to antagonize the action of isoniazid; these substances were tested as antagonists of the inhibition by isoniazid of acetate oxidation by suspensions of B.C.G.

Vitamin B₆. Yoneda, Kato & Okajima (1952) reported that the formation of indole by *Escherichia coli communius* was inhibited by isoniazid, and that this inhibition was reversed by pyridoxin or pyridoxal. Yoneda & Asano (1953) reported that the arginine decarboxylase of *E. coli communius* was also inhibited by isoniazid and that the inhibition was annulled by pyridoxal and pyridoxin. It was suggested that the similarity of their chemical structure (presence of the pyridine nucleus) might be the cause of antagonism between pyridoxin and its derivatives, on the one hand, and isoniazid.

Pyridoxin (10^{-2} to 10^{-6} M) and pyridoxal (10^{-3} to 10^{-6} M) were used in an attempt to antagonize the inhibition of acetate oxidation by isoniazid. Neither pre-treatment of the suspensions of B.C.G. with pyridoxin or pyridoxal, nor adding the vitamin with isoniazid produced any antagonism of isoniazid inhibition. Growth experiments also showed no change in the sensitivity of B.C.G. to isoniazid whether grown in the presence or absence of vitamin B₆ and its derivatives.

Manganous chloride was reported by Fisher (1954*a*) to annul the inhibitory action of isoniazid on the growth of *Mycobacterium tuberculosis* H37Rv at a concentration of 0.01 %. No such effect was demonstrated with B.C.G. suspensions oxidizing acetate in the Warburg apparatus.

Biotin. Pitillo & Foster (1954) reported that the inhibition by isoniazid of growth of mycobacterium A.F. 2 in broth culture was reversed by the addition of biotin to the medium. Biotin (final concentration 1 µg./ml.) was therefore added with isoniazid to suspensions of B.C.G. oxidizing acetate, but without effect.

Oleic acid derivatives. In view of the role of biotin in the synthesis of oleic acid (Williams, Broquist & Snell, 1947) it was thought that isoniazid might block fatty acid production. Sodium oleate (10^{-2} M) and Tween 80 (a polyoxyethylene derivative of sorbitan mono-oleate, 0.02 %), were each added to the acetate system. Neither of these substances was oxidized, nor did they have any effect on the isoniazid inhibition.

Haemin. The antagonism of isoniazid inhibition by haemin was first noticed by Fisher (1954*a, b*), following his discovery that some isoniazid-resistant strains in certain media required haemin as a growth factor. Under certain conditions alkaline haemin combines with isoniazid (Cohn, Oda, Kovitz & Middlebrook, 1954; Knox, Albert & Rees, 1955); and Gray (1953) suggested that inhibition of growth by isoniazid depends on its combination with essential porphyrin-containing enzymes, which are present in mycobacteria in very low concentration. Haemin was tested at concentrations up to 10^{-2} M as an antagonist to isoniazid in the Warburg apparatus, both by direct addition to suspensions and by growing the B.C.G. in the presence of haemin. There was no antagonism of the inhibitory effect of isoniazid, nor did haemin stimulate

basal oxygen uptake, nor oxygen uptake in the presence of acetate, with sensitive or resistant strains of B.C.G.

Shemin (1948) showed that glycine is involved in the synthesis of porphyrins by avian erythrocytes *in vitro*; Lascelles (1955) demonstrated the synthesis of porphyrins from glycine and α -ketoglutaric acid by suspensions of *Rhodospirillum spheroides*. These substances were therefore added, together or singly, in the presence and absence of isoniazid, to the acetate oxidizing system, in an attempt to annul isoniazid inhibition by allowing for the synthesis of haemin during the experiment. Glycine and α -ketoglutaric acid did not stimulate oxygen uptake, nor did they prevent inhibition of acetate oxidation by isoniazid.

Catalase. Middlebrook, Cohn & Schaefer (1954) showed that isoniazid-resistant strains were deficient in catalase, and it was thought possible that isoniazid inhibition might involve catalase activity. Catalase was tested as an antagonist of isoniazid inhibition of the acetate system but had no effect. Haemin and bovine albumin fraction V 0.002 % (w/v), singly or together, were added to B.C.G. suspensions oxidizing acetate. These substances did not increase oxygen uptake, or prevent inhibition by isoniazid.

Effect of disinfectants on acetate oxidation by B.C.G. suspensions

The effect of other antibacterial substances on the oxidation of acetate by washed suspensions of B.C.G. was tested. All the disinfectants tested were capable of causing complete inhibition of acetate oxidation. The inhibitory concentration required was the same for isoniazid-sensitive and isoniazid-resistant strains of B.C.G. (Table 5).

Table 5. *Disinfectants and acetate oxidation by B.C.G.*

Disinfectant	Concentration required for complete inhibition
	(%, w/v)
Bradosol	0.5
Comprox A	0.05
Phenol	0.01
Cetavlon	0.001

Mixtures of growth inhibitory compounds

In view of reports of delayed emergence of resistant strains in clinical tuberculosis by the simultaneous administration of two antituberculous drugs, the effect on acetate oxidation by B.C.G. of mixtures of drugs was tested. It was found that in certain cases mixtures of drugs behaved synergistically, i.e. concentrations of drugs which alone caused no inhibition of acetate oxidation, exerted an inhibitory effect when added with subinhibitory concentrations of another drug.

The maximum inhibition of acetate oxidation obtained with any of the drugs tested was approximately 50 %; the effective drugs were isoniazid and

streptomycin. Aureomycin, terramycin, achromycin, *p*-aminosalicylic acid (P.A.S.), thiosemicarbazone (T.B.1) and cyanacetic acid hydrazide (C.A.H.) had no inhibitory or stimulatory effect on the oxidation of acetate by the sensitive strain of B.C.G. As shown in Table 6, mixtures of subinhibitory concentrations of isoniazid+streptomycin, P.A.S. or terramycin, or streptomycin+P.A.S. gave a 50 % inhibition of acetate oxidation. The mixtures isoniazid+C.A.H., T.B.1, aureomycin or achromycin were ineffective.

Table 6. *Effect of various mixtures of anti-tuberculous drugs on oxidation of acetate by B.C.G. (S)*

Drug	Concentration of drug used (M)	Isoniazid concentration			Streptomycin 2×10^{-2} M
		0	10^{-4} M	5×10^{-4} M	
—	—	0	0	+	0
Streptomycin	2×10^{-1}	+	+	+	
	4×10^{-2}	+	+	+	
	2×10^{-2}	0	+	+	
P.A.S.	1	0	+	+	0
	10^{-1}	0	+	+	+
	10^{-2}	0	+	+	+
Terramycin	10^{-3}	0	+	+	0
Aureomycin	10^{-3}	0	0	+	0
Achromycin	10^{-3}	0	0	+	0
T.B.1	2×10^{-1}	0	0	+	0
	2×10^{-2}	0	0	+	0
	2×10^{-3}	0	0	+	0
C.A.H.	1	0	0	+	0
	10^{-1}	0	0	+	0
	10^{-2}	0	0	+	0

+ = 47–54 % inhibition of acetate oxidation; 0 = no inhibition of acetate oxidation.

DISCUSSION

The effect of isoniazid on acetate oxidation by suspensions of B.C.G. as described above has several characteristics in common with its effect on the growth of mycobacteria. At low concentrations isoniazid is effective in inhibiting the growth of mycobacteria only, other organisms being unaffected. Similarly, of the organisms tested, only members of the genus *Mycobacterium* show inhibition of acetate oxidation by isoniazid. The concentration of isoniazid required to inhibit the growth of mycobacteria depends on inoculum size and the particular strain used. The growth of various strains of *M. tuberculosis* (both human and bovine) is inhibited by less isoniazid than the saprophytic mycobacteria such as *M. smegmatis* and *M. phlei*. In acetate oxidation the same conditions obtain; large cell concentrations require more isoniazid for inhibition than smaller ones, and *M. tuberculosis* (H37Ra and B.C.G.) is much more sensitive to isoniazid than *M. smegmatis*. Similarly, strains of *M. tuberculosis*, which are resistant to isoniazid in growth, are also resistant to it by the acetate oxidation test. In this respect B.C.G. differs from *M. smegmatis*; the amount

of isoniazid required to inhibit oxidation of acetate by *M. smegmatis* is not related to its sensitivity to isoniazid (Meadow, 1956).

So far it has not proved possible to determine the particular stage of acetate oxidation which is inhibited by isoniazid since the reaction has never gone to completion. By using lower concentrations of acetate and more concentrated suspensions of organisms it was hoped to achieve this effect. However, decrease of acetate concentration also decreased the rate of reaction to a marked extent and prevented the oxidation from going to completion. Similarly, increases in suspension densities increased the amount of isoniazid required for inhibition so that the range of the experiment was limited by the solubility of isoniazid.

Inhibition of pyruvate oxidation by isoniazid is probably due to chemical combination between pyruvate and isoniazid, thus effectively removing some pyruvate from the system. The hydrazone formed between isoniazid and pyruvate when tested on the system had no effect; it was not oxidized by suspensions of B.C.G. nor did it inhibit acetate oxidation. It seems unlikely, however, that isoniazid and acetate combined chemically since there was no disappearance of isoniazid from the supernatant fluid after use in the Warburg apparatus, as measured by ultraviolet spectroscopy. It appears, therefore, that this isoniazid inhibition of acetate oxidation by B.C.G. may represent fairly closely the conditions in growth inhibition.

The effectiveness of mixtures of subinhibitory concentrations of anti-tuberculosis drugs in inhibiting the oxidation of acetate bears a resemblance to the clinical results obtained by the simultaneous administration of two drugs. The effective mixtures of drugs in the treatment of certain types of pulmonary tuberculosis appear to be: isoniazid + streptomycin, streptomycin + P.A.S., isoniazid + P.A.S. (Joiner *et al.* 1952, 1953; Medical Research Council, 1952, 1953*a, b*); isoniazid + terramycin (Stewart, Turnbull & Crofton, 1954). These are also the mixtures which are effective in the Warburg apparatus. It has been suggested that the usefulness of mixtures of drugs in the treatment of tuberculosis is partly due to suppression of the emergence of resistant strains. However, the results obtained in the Warburg apparatus indicate that another explanation must be sought, since the 6 hr. duration of the experiments would not be sufficient to allow the emergence of a resistant strain, whether or not antituberculous drugs were present. In these particular mixtures of drugs it appears that there is a genuine synergism between the members of the pairs.

The most puzzling of the results obtained concern haemin. Haemin has been shown (Knox, Albert & Rees, 1955; Albert & Rees, 1955; Knox & Woodroffe, 1955) to react with isoniazid, converting it to di-isoniazid and isonicotinic acid, neither of which compounds inhibits the growth of mycobacteria except at high concentrations. *Mycobacterium tuberculosis* H37Rv grows in the presence of 8 μ g. di-isoniazid/ml. or 80 μ g. isonicotinic acid/ml. The conversion of isoniazid to di-isoniazid or to isonicotinic acid would therefore effectively decrease the concentration of inhibitory substances present and might explain the annulment of isoniazid inhibition by haemin in growth. No such antagonism of inhibition was detected by adding haemin to the system which showed inhibition by isoniazid of acetate oxidation. This may have been due to the

short duration of the experiment. Even under optimum conditions for isoniazid oxidation (shaking in buffer pH 7.5 with excess haemin) only one-third of the isoniazid present was oxidized in 24 hr. The concentration of haemin which may be added to the system in Warburg flasks is limited by its relative insolubility in phosphate buffer. Perhaps if a higher concentration could be used, the rate of reaction would be increased and some antagonism of isoniazid inhibition be detected.

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Some Factors Affecting Lactase Formation and Activity in *Saccharomyces fragilis*

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SUMMARY: A method for determining the lactase activity of *Saccharomyces fragilis* is described. The yeast is treated with cetyltrimethylammonium bromide, and the products of lactose hydrolysis are fermented with *S. mandshuricus*. The formation of lactase in *S. fragilis* was studied by using the continuous culture technique. Lactase formation was inhibited by the presence of sugars in the medium at concentrations greater than 0.001 % (w/v), but the magnitude of the inhibition and the range of activity over which it occurred depended on the nature of the sugar in the medium. With media containing glucose or sucrose, activities up to 10 units lactase/mg. dry weight were found when the sugar concentration was less than 0.001 % (w/v), while at high concentrations lactase activity was almost completely absent. With media containing galactose or lactose at concentrations less than 0.001 % (w/v) activities of approximately 100 units lactase/mg. dry weight were observed, while at concentrations greater than 0.01 % (w/v) the activity was less. The mean generation time of the organisms and the concentrations of growth factors, ammonium and hydrogen ions had, over the range tested, no significant effect on lactase formation. The lactase activity found in intact organisms was always lower than the activity found in disrupted organisms, irrespective of the conditions under which the yeast had been grown. Possible interpretations of this phenomenon are discussed.

Lactase was isolated from a strain of *Saccharomyces fragilis* by Caputto, Leloir & Trucco (1948) and its properties studied. They found that its optimum pH value was 6.8 and that it was activated by manganese, magnesium or potassium ions. Roberts & McFarren (1953) studied the products of *S. fragilis* lactase action on lactose and found several oligosaccharides as well as glucose and galactose. R. Davies (unpublished results) found that a partially purified lactase preparation was activated by potassium ion and to a lesser extent by sodium ion, and that the activation by potassium ion was depressed by sodium ion. The latter worker also found that the lactase was unstable in water but fairly stable in solutions of potassium salts, the greatest stability being found in potassium phosphate buffer. In contrast to the lactase of *Escherichia coli*, however, little work appears to have been performed on the conditions which govern the formation of this enzyme in *S. fragilis*.

The advantages of the continuous culture technique for studying enzyme formation in micro-organisms have been described in a previous paper (A. Davies, 1956). The present paper describes investigations on the effect of certain sugars and other carbon sources on lactase formation in *Saccharomyces fragilis*. The effects of the mean generation time of the organism, the concentrations of ammonium and hydrogen ions and of growth factors have also been studied.

METHODS

Organisms. *Saccharomyces fragilis* Jörgensen was obtained from the Centraal-bureau voor Schimmelcultures, Delft; *Saccharomyces mandshuricus* (Saito, 1916) from the Carlsberg Laboratory, Copenhagen; *Torulopsis pulcherrima* from the National Collection of Type Cultures, London.

Growth conditions and analytical procedures. The preparation of the media and suspensions of organisms, analysis of growth media and the method of use of the chemostat were as described by Davies (1956). *Saccharomyces mandshuricus* for use in the estimation of lactase activity was grown for 18 hr. at 30° in Roux bottles in the basal medium of Davies, Falkiner, Wilkinson & Peel (1951) + 2 % (w/v) galactose.

Determination of lactase activity. R. Davies (unpublished results) found that lactase activity of *Saccharomyces fragilis* was increased following certain treatments which either disrupted the organisms or damaged the cell wall. In order to follow the effect of various growth conditions on the lactase content of *S. fragilis* it was therefore necessary to measure the enzymic activity under conditions which would eliminate the masking effect of the cell wall. The method adopted depends on the fact that galactose-adapted *S. mandshuricus* is unable to ferment lactose but ferments quantitatively both the products of lactose hydrolysis. The detergent cetyltrimethylammonium bromide (CTAB) rapidly 'unmasks' the full lactase activity of *S. fragilis*, but the enzyme is rapidly denatured. However, the rate of denaturation is considerably decreased in a medium which contains a high concentration of lactose and potassium phosphate. On addition of *S. mandshuricus* the excess of CTAB is removed and gas evolution remains steady for long periods. Salton (1951) showed that bacteria can absorb up to half their dry weight of CTAB, though much smaller quantities exert a maximal effect on release of low molecular weight components from the cell. The action of detergents on micro-organisms seems to be dependent on the ratio of weight of detergent to weight of micro-organism rather than on the concentration of the detergent (Gale & Taylor, 1947, Salton, 1951). This explains why it is possible to disrupt *S. fragilis* with CTAB, a procedure which completely inhibits its fermentation system, and then by employing large amounts of *S. mandshuricus* to remove the excess CTAB from solution without seriously affecting the fermentation rate of the latter yeast.

The method used for routine determinations of activity was as follows: 0.3 ml. of a suspension of *Saccharomyces fragilis* was placed in the main compartment of a double side-arm Warburg manometer vessel, together with 0.2 ml. of M-potassium phosphate buffer (pH 6.0). One ml. of 10 % (w/v) lactose and 0.25 ml. of 0.4 % (w/v) CTAB were placed in one side-arm; 1.25 ml. of *S. mandshuricus* preparation were placed in the other side-arm. The latter preparation consisted of 25 ml. of a washed, aqueous suspension of *S. mandshuricus* (100 mg. dry wt. organisms/ml.) to which 5 ml. M-potassium phosphate buffer (pH 6.0) and 1 ml. 0.1 M-sodium azide were added; the preparation was then shaken for 3 hr. at 25° to decrease the endogenous fermenta-

tion. After equilibration at 25° the CTAB and lactose were tipped into the main compartment, the solutions thoroughly mixed and the *S. mandshuricus* preparation tipped a few seconds later. The variation between replicate estimations was $\pm 5\%$. Lactase activity is expressed as μ mole lactose hydrolysed/mg. dry wt. organisms/hr. The unit of enzyme is that amount of enzyme which will hydrolyse 1 μ mole lactose in 1 hr. at 25°.

To check the results obtained by this procedure a second method was developed. Samples of *Saccharomyces fragilis* were measured into manometer cups and dried for 2 hr. over phosphorus pentoxide *in vacuo* (Lederberg, 1950). Phosphate buffer and the *S. mandshuricus* preparation were then added to the dried yeast and 1 ml. 10% (w/v) lactose placed in the side-arm. The carbon dioxide evolved after tipping was measured at 25°. The activities obtained using this method varied from 80 to 100% of those observed when the CTAB procedure was used.

Fermentation rates were determined in Warburg manometers at 25° according to the techniques of Umbreit, Burris & Stauffer (1949), and were expressed in terms of $Q_{CO_2}^{N_2} = \mu$ l. CO_2 /mg. dry wt./hr.

Chemicals. Kerfoots Biochemical Reagent Sugars were used throughout; other chemicals were of analytical quality.

RESULTS

Lactase formation during growth in shaken flasks

The change in lactase content of *Saccharomyces fragilis* with time of growth under various conditions is shown in Fig. 1. Curves marked *A* show lactase formation and growth in a medium initially containing 1.5% (w/v) glucose, 352 mg. ammonia-N/l. and 20% of the standard amount of growth factors. The lactase content of the organisms remained low until growth had almost ceased and then rose rapidly to 4 units/mg. This rise in activity was concurrent with the disappearance of glucose from the medium. When the yeast was grown in a similar medium containing 0.1% (w/v) glucose, cell division ceased earlier and at a lower culture density than in the previous case. The lactase content was low at the beginning of the experiment and rose to approximately 3 units/mg. during growth (Fig. 1, curves *B*).

With a medium containing 2% (w/v) glucose, 105 mg. ammonia-N/l. and 20% of the standard concentration of growth factors, the culture density was decreased compared to a culture grown on a medium containing 352 mg. ammonia-N/l. The yeast took 39 hr. to exhaust the medium of its glucose. In these conditions the lactase content was low for most of the experiment (Fig. 1, curves *C*).

When growth factors were present in the medium at the standard concentrations, glucose utilization ceased when growth ceased. When *S. fragilis* was grown in a medium containing growth factors at the standard concentrations, 2% (w/v) glucose and either 105 or 352 mg. ammonia-N/l. the lactase content remained at a low level throughout the course of the experiment.

When the yeast was grown in the basal medium (352 mg. ammonia-N/l.,

60 % of the standard concentration of growth factors) plus 2 % (w/v) galactose, the lactase content rose during the early phases of growth, reaching 110 units/mg. when the culture was 18 hr. old, i.e. about the end of the exponential phase, and then diminished towards the end of growth (Fig. 2).

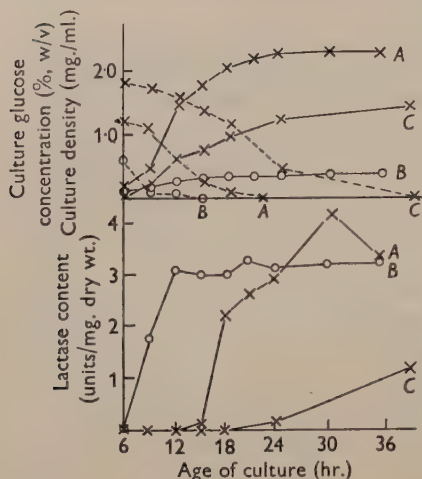


Fig. 1

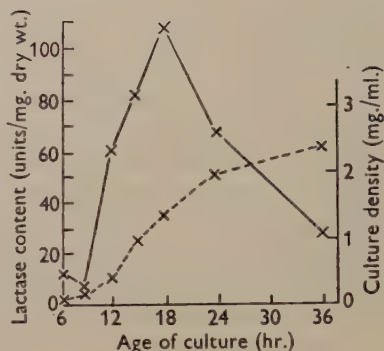


Fig. 2

Fig. 1. The effect on lactase content of *Saccharomyces fragilis* of growth for different periods of time in media containing various amounts of glucose and nitrogen. In top graph continuous lines = culture density, broken lines = culture glucose concentration. Curves A: 1.5 % (w/v) glucose, 352 mg. ammonia-N/l., 20 % of standard amounts of growth factors. Curves B: 0.1 % (w/v) glucose otherwise as curves A. Curves C: 2.0 (w/v) glucose, 105 mg. ammonia-N/l., 20 % of the standard concentrations of growth factors. In curves B the glucose concentration is plotted at 10 times its actual concentration for clarity.

Fig. 2. The effect on lactase content of growth for different periods of time in a medium containing 2.0 % (w/v) galactose, 352 mg. ammonia-N/l. and 60 % of the standard amounts of growth factors. The continuous line represents the lactase content, the broken line represents the culture density.

Lactase formation during growth in the chemostat

Media containing glucose. When *Saccharomyces fragilis* was grown in a chemostat supplied with medium initially containing 9.5 % (w/v) glucose, 105 μ g. ammonia-N/ml. and standard concentrations of growth factors, the glucose concentration in the culture became steady at approximately 8 % (w/v), the nitrogen at approximately 20 μ g./ml. and the lactase content of the resultant organisms was less than 0.1 unit/mg. dry wt. at all mean generation times. Similar results were obtained when the reservoir medium contained 2 % (w/v) glucose; in this case the glucose concentration in the culture was always greater than 0.5 % (w/v); the nitrogen concentration was less than 3 μ g./ml. for all flow rates except that corresponding to a mean generation time of 1.6 hr. where it rose to 7 μ g./ml. In Fig. 3 lactase contents are plotted against mean generation time for organisms grown in media initially containing 0.5, 0.25

and 0.1% (w/v) glucose. In these cases the activity varied with the mean generation time but it could also be correlated with the glucose concentrations in the cultures. In Fig. 4, (compiled from the results of a large number of experiments) the lactase content is plotted against the logarithm of the glucose concentration in the culture for mean generation times of 3 hr. and 10 hr. The two curves are substantially the same, showing that the rate of cell division had little effect on lactase biosynthesis under these conditions. It would appear that lactase formation only occurred to a significant extent in media containing less than 0.001% (w/v) glucose.

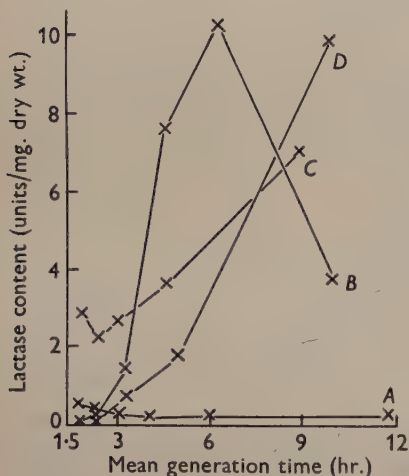


Fig. 3

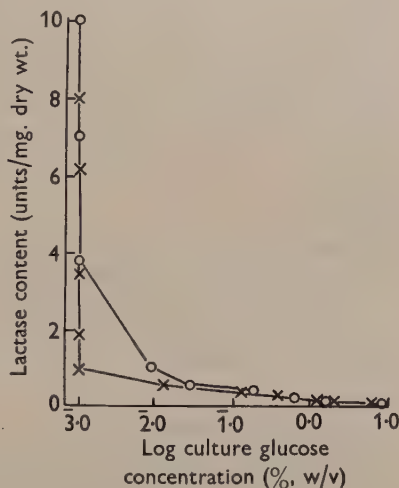


Fig. 4

Fig. 3. Variation of lactase content with mean generation time. Initial glucose concentrations (% w/v) in basal medium: A, 0.5; B, 0.25; C, 0.1. Curve D, 0.5% (w/v) glucose, ammonia-N concentration of basal medium increased from 105 to 352 mg./l.

Fig. 4. Effect of culture glucose concentration and mean generation time on lactase content. Cells grown in basal medium + glucose with mean generation times of: 3 hr. (x—x) and 10 hr. (o—o). The limit of sensitivity of the sugar analysis procedure is 0.001% (w/v) glucose. Growth limited by nitrogen at glucose concentrations greater than 0.001%, growth limited by glucose at glucose concentrations of 0.001% or less.

Effect of growth factors and nitrogen concentration. Growth in the chemostat can be controlled by decreasing the concentrations of growth factors. It is then possible to study the effect of growth factor deficiency and nitrogen and glucose excess. It was found that variations in nitrogen concentration or the nature of the growth factor which was limiting had little effect on the lactase content. The overriding effect of the steady-state glucose concentration in the culture is again shown in Fig. 5 in which the logarithm of the culture glucose concentration is plotted against the lactase content for the two cases (a) where the nitrogen concentration was less than $2\mu\text{g./ml.}$, (b) where it was greater than $10\mu\text{g./ml.}$ The two curves are almost identical at glucose concentrations greater than 0.001% (w/v) which is the limit of sensitivity of the sugar determination procedure. The hump on the second curve at 0.6% (w/v)

glucose was obtained at long mean generation times when growth was limited by biotin deficiency. Since this rise in activity did not occur when growth was limited by deficiency of other growth factors, it is probably due to the specific conditions of growth under biotin deficiency at long mean generation times and not to the presence of excess nitrogen in the culture.

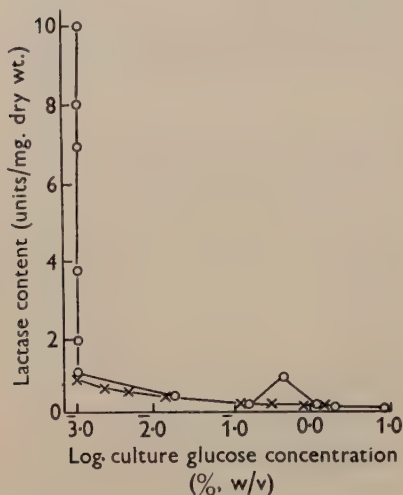


Fig. 5

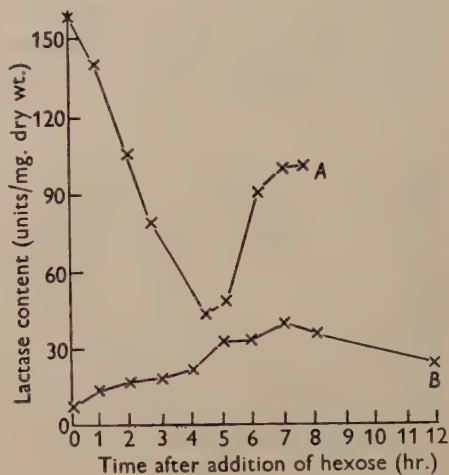


Fig. 6

Fig. 5. Effect of culture glucose concentration and nitrogen concentration on lactase content. Cells grown in basal medium + glucose. \times — \times , culture containing < 2 mg. ammonia-N/l., growth limited by nitrogen; o — o , culture containing > 10 mg. ammonia-N/l., growth limited by growth factors at glucose concentrations greater than 0.001%, growth limited by glucose at glucose concentrations of 0.001% or less.

Fig. 6. Curve A: effect of adding glucose at a final concentration of 1.0% to a culture growing on basal medium + 0.2% (w/v) galactose with a mean generation time of 3.7 hr. Curve B: effect of adding galactose to a final concentration of 1.0% (w/v) to a culture growing on basal medium + 0.1% (w/v) glucose with a mean generation time of 3.5 hr.

Effect of the pH value of growth medium. The pH value of cultures in the chemostat varied between pH 2.6 and 3.4, depending on the concentration of sugar in the medium. However, by adding appropriate amounts of potassium hydroxide to the medium it was possible to grow the yeast over a wide range of pH values in media of otherwise similar composition. When the basal medium contained 0.1% (w/v) glucose and the mean generation time was 4 hr., the lactase content increased from 8 to 11 units/mg. as the culture pH value was increased from 3.4 to 7.7. This difference was only twice that obtained when yeast was grown under constant cultural conditions in a 0.1% (w/v) glucose medium. When the pH value of the culture was increased from 7.7 to 8.3 the lactase content decreased from 11 to 1.2 units/mg. This decrease could be accounted for by the increase in the culture glucose concentration from undetectably low concentrations at pH 7.7 to 0.014% (w/v) at pH 8.3. It would appear that the pH value *per se* had little effect on the lactase content under conditions in the chemostat.

When growth occurred on a 2% (w/v) glucose medium at a mean generation time of 3.5 hr. the lactase content increased from 0.1 unit/mg. at pH 2.6 to 1.0 unit/mg. at pH 7.2. When the activities found in the 0.1% (w/v) glucose culture are compared with those found in the 2% (w/v) glucose culture it is clear that the inhibitory effect of glucose on lactase formation is present at all pH values.

Growth in the chemostat in media containing sugars other than glucose

Sucrose. When *Saccharomyces fragilis* was grown in the basal medium + 1% (w/v) sucrose the lactase content varied from 0.03 to 0.2 unit/mg., while when growth occurred in the basal medium initially containing 0.2% (w/v) sucrose the lactase content decreased from 8.7 units/mg. at a mean generation time of 8.3 hr. to 0.6 unit/mg. at a mean generation time of 1.6 hr. These values of lactase content could be correlated with the variations in concentration of invert sugar in the culture (Table 1).

Table 1. *Correlation of lactase content and invert sugar in the culture medium for Saccharomyces fragilis grown in basal medium + sucrose*

Saccharomyces fragilis was grown in chemostat in basal medium + initially 0.2% (w/v) sucrose. Lactase content determined by measuring carbon dioxide production from lactose in presence of *S. mandshuricus* and CTAB-treated *S. fragilis*. Invert sugar concentration determined by the method of Davies (1956).

Mean generation time (hr.)	8.3	4.4	2.75	2.15	1.60
Lactase content (units/mg. dry wt. <i>S. fragilis</i>)	8.7	3.0	2.5	1.0	0.6
Invert sugar concentration (% w/v)	<0.001	<0.001	0.002	0.004	0.01

Table 2. *Correlation of lactase content and sugar concentration in the culture for Saccharomyces fragilis grown in the chemostat in basal media + lactose*

Sugar concentrations were determined by fermentation with *Saccharomyces fragilis* (Davies, 1956). Lactase was estimated as in Table 1.

(a) Medium initially containing 1% (w/v) lactose

Mean generation time (hr.)	1.70	2.20	3.4	5.3	13.8
Lactase content (units/mg.)	21	27	35	19	17
Sugar concentration (% w/v)	0.60	0.43	0.10	0.26	0.09

(b) Medium initially containing 0.2% (w/v) lactose

Mean generation time (hr.)	1.70	2.20	2.90	4.9	10.6
Lactase content (units/mg.)	28	32	56	65	100
Sugar concentration (% w/v)	0.008	0.013	0.004	<0.001	<0.001

Lactose and galactose. The values of lactase content found in *Saccharomyces fragilis* grown in basal media + lactose or galactose showed a twofold to tenfold increase over those found in basal media + growth-limiting amounts of glucose. This suggests, but does not prove, that lactose and galactose induced lactase formation by this organism. In Table 2 the relation between lactase content

and residual sugar in the culture is shown for growth on basal media containing 1.0 % and 0.2 % (w/v) lactose at various mean generation times, and it will be seen that the lactase content decreased as the steady state concentration of sugar in the medium increased. The nature of the sugar in the medium was not determined in either of these experiments, but evidence is presented in a later paragraph that it consisted of a mixture of lactose, glucose and galactose. It is not possible to state whether lactose itself has any inhibitory effect on lactase formation since the glucose in the culture might account for the observed reduction in enzymic activity. From a comparison of the activities found in the cultures grown in the 0.2 % (w/v) and the 1.0 % (w/v) lactose media it would appear that a concentration of sugar equivalent to 0.01 % (w/v) lactose in the culture exerted an almost maximal inhibitory effect on

Table 3. *Correlation of lactase content and galactose concentration in the culture for yeast grown in the chemostat in basal media + galactose*

Sugar concentrations were determined by fermentation with *Saccharomyces fragilis* (Davies, 1956). Lactase was estimated as in Table 1.

(a) Medium initially containing 1.0 % (w/v) galactose					
Mean generation time (hr.)	1.70	2.35	3.2	5.6	9.8
Lactase content (units/mg.)	31	39	40	33	40
Sugar concentration (% w/v)	0.74	0.52	0.49	0.36	0.21
(b) Medium initially containing 0.2 % (w/v) galactose					
Mean generation time (hr.)	1.70	2.40	3.2	5.2	10.3
Lactase content (units/mg.)	41	82	102	101	126
Sugar concentration (% w/v)	0.05	0.004	0.002	<0.001	<0.001

lactase formation. A similar dependence of lactase activity on sugar concentration was found for growth in galactose media under similar conditions (Table 3). Since the lactase content did not vary unduly with mean generation time for growth on both the 1.0 % (w/v) lactose and the 1.0 % (w/v) galactose media, it would appear that the rate of cell division had little effect on the formation of lactase.

Mixtures of glucose and galactose. The effect of adding glucose to an exponentially growing culture of *Saccharomyces fragilis* which had a high lactase content was demonstrated by the following experiment. From a culture growing with a mean generation time of 3.7 hr. in the basal medium + 0.2 % (w/v) galactose, a 10 ml. sample was collected. Glucose was then added directly to the culture to give a final concentration of 1.0 % (w/v). Further samples were collected at intervals and their lactase contents determined. There was an immediate and rapid decrease in the lactase content followed by a rise (Fig. 6). This experiment indicated that under these poorly regulated conditions an excess of glucose exerted an inhibitory effect on lactase formation. This diminution in activity does not necessarily imply that enzyme destruction was occurring, since a decrease in the activity of organisms collected from the chemostat would occur by a cessation of enzyme production coupled with continued growth.

The effect of adding galactose to a final concentration of 1.0 % (w/v) to a culture growing exponentially (mean generation time 3.5 hr.) in a 0.1 % (w/v) glucose medium is shown in Fig. 6 (curve *B*). An increase of lactase content was observed. Thus galactose, in the presence of glucose, stimulated the formation of lactase.

Table 4. *Effect on lactase content and lactose fermentation of growth of Saccharomyces fragilis in a medium containing various amounts of glucose and galactose*

The organisms were grown in the chemostat with a mean generation time of 3.3 hr. Lactase estimated as in Table 1.

Input glucose (% w/v)	0.2	0.199	0.198	0.193	0.183	0.167
Input galactose (% w/v)	0.0	0.001	0.002	0.007	0.017	0.033
Lactase content (units/mg.)	6.5	9.4	11.1	15.7	27.5	39.0
Lactase content (expressed as Q_{CO_2})	580	840	1000	1400	2500	3500
Lactose fermentation rate ($Q_{CO_2}^{N_2}$)	5.5	13	19	43	43	53
Glucose fermentation rate ($Q_{CO_2}^{N_2}$)	22	24	32	56	50	52
Input glucose (% w/v)	0.2	0.18	0.15	0.11		
Input galactose (% w/v)	0.0	0.02	0.05	0.09		
Lactase content (units/mg.)	5.7	36	64	102		
Lactase content (expressed as Q_{CO_2})	510	3200	5600	9200		
Lactose fermentation rate ($Q_{CO_2}^{N_2}$)	8.0	50	43	40		
Glucose fermentation rate ($Q_{CO_2}^{N_2}$)	30	47	46	36		

By arranging two supplies of medium into one chemostat it was possible to obtain media containing various ratios of glucose and galactose, whilst maintaining the total hexose and all other constituents of the medium constant. The results are given in Table 4. With a medium containing 0.007 % (w/v) galactose in a total hexose concentration of 0.2 % (w/v) a 250 % increase in lactase content over the value found in a 0.2 % (w/v) glucose medium was observed. The lactase content steadily increased as the ratio of glucose to galactose was decreased. With a medium initially containing 0.09 % (w/v) galactose and 0.11 % (w/v) glucose the lactase content reached 102 units/mg., a value not much smaller than that observed in a 0.2 % (w/v) galactose medium. When galactose was added to cultures growing in media containing glucose in concentrations greater than 0.2 % (w/v) little effect on the lactase content was observed (Table 5).

Growth in other carbon sources. Because of the low growth rates of *Saccharomyces fragilis* in the presence of the compounds examined (Davies, 1956) cultures were grown in Roux bottles in basal medium + 1 % (w/v) of the sodium salts of the substances listed below as carbon sources and the lactase content determined after various periods of growth.

With *acetate* the lactase content varied from 7.2 to 8.7 units/mg. This variation is not significant. With *citrate* the lactase content increased from

3.1 units/mg. in the early phases of growth to a maximum of 7.7 units/mg. 24 hr. after maximum growth was obtained and then decreased to 4.8 units/mg. With *lactate* the lactase content decreased from 2.9 units/mg. in 'young' (18 hr.) cultures to 1.6 units/mg. in the phase of exponential growth and then increased again when growth ceased. With *succinate* the lactase content increased from 3.2 units/mg. at the beginning of growth to a maximum of 5.4 units/mg. when maximum growth was attained. With 1% (v/v) *ethanol* the lactase content was 8–10 units/mg. in cultures in the lag and early logarithmic phases of growth, then steadily decreased to 1 unit/mg. during the later stages of growth. With *glycerol* (1%, w/v) the lactase content remained steady at 6.6 units/mg. until growth ceased when there was a decrease to 1.3 units/mg.

Table 5. *Effect of ratio of glucose to galactose on lactase content*

Saccharomyces fragilis was grown in the chemostat in basal medium + various ratios of glucose to galactose. Culture hexose was estimated by fermentation with *S. fragilis*. Lactase estimated as in Table 1.

Mean generation time (hr.)	Initial glucose (% w/v)	Initial galactose (% w/v)	Culture hexose (% w/v)	Lactase content (units/mg.)
3.3	2.0	0.0	1.2	0.03
3.4	1.87	0.013	0.80	0.1
3.3	1.67	0.033	0.64	0.03
3.3	0.86	0.11	0.12	1.1
3.3	0.62	0.14	0.13	0.1

Lactose fermentation and hydrolysis by intact Saccharomyces fragilis

When *Saccharomyces fragilis* was grown in the basal medium + galactose or lactose in either the chemostat or in Roux bottles, the rate of lactose fermentation was approximately equal to the rate of glucose fermentation, irrespective of the concentration of sugar in the medium or the rate of division (Table 6).

Table 6. *Fermentation rates for different sugars by Saccharomyces fragilis*

Yeast grown in the chemostat at two different mean generation times in basal medium + lactose or galactose.

Initial sugar concentration in medium	$Q_{\text{CO}_2}^{\text{N}_2}$ (glucose)	$Q_{\text{CO}_2}^{\text{N}_2}$ (lactose)	$Q_{\text{CO}_2}^{\text{N}_2}$ (galactose)
0.2% lactose			
(a)	110	110	8
(b)	70	60	10
1.0% lactose			
(a)	55	40	9
(b)	125	120	65
0.2% galactose			
(a)	35	30	20
(b)	60	50	15
1.0% galactose			
(a)	75	65	20
(b)	175	150	55

(a)=organisms grown at c. 10 hr. mean generation time. (b)=organisms grown at c. 1.7 hr. mean generation time.

When *S. fragilis* was grown in media containing an excess of glucose, lactose was not fermented. When the yeast was grown with a mean generation time of 3.3 hr. in a medium initially containing 0.2 % (w/v) glucose, the organisms possessed lactase contents of 5.7, 6.1, 6.4, 6.5 and 6.6 units/mg., respectively, in five separate experiments. If the fermentation rates were determined by the lactase content these values would correspond to $Q_{CO_2}^{N_2}$ (lactose) = 510, 550, 570, 580, 590. The observed values for $Q_{CO_2}^{N_2}$ (lactose) were 8.0, 6.3, 6.3, 5.5, 7.0. The $Q_{CO_2}^{N_2}$ (glucose) values for these organisms were 30, 32, 49, 22, and 26, respectively.

Partial replacement of glucose by galactose (even at low concentration) in a medium initially containing 0.2 % (w/v) glucose resulted in an increased rate of fermentation of lactose and of lactase activity (Table 4).

That the products of lactase action on lactose are glucose and galactose was demonstrated in the following way. Washed organisms from a culture of *Saccharomyces fragilis* grown on a medium containing 2 % (w/v) lactose were dried over phosphorus pentoxide for 2 hr. *in vacuo*, resuspended in phosphate buffer and incubated with 3 % (w/v) lactose for 30 min. at 25°. The yeast was removed by centrifugation and the sugars in the supernatant separated by single dimensional paper chromatography on Whatman no. 1 paper using ethyl acetate/pyridine/water (2:1:2) (Jermyn & Isherwood, 1949) as the solvent. The chromatograms were developed for 15 hr. at room temperature, dried, and the positions of the sugars located with aniline hydrogen phthalate (Partridge, 1949). Sugars with R_F values corresponding to lactose, glucose and galactose markers were found on the chromatogram; no other sugars were found.

Since *Saccharomyces fragilis* grown on a medium containing limiting concentrations (< 0.25 %, w/v) of glucose possessed lactase contents of up to 10 units/mg. (equivalent to $Q_{CO_2}^{N_2}$ = 900), yet was unable to ferment lactose with a $Q_{CO_2}^{N_2}$ value greater than 8.0, although the rate of glucose fermentation was approximately four times this value, it would appear that the lactase was almost inactive in the intact organisms. A similar situation exists in organisms grown in media containing galactose or lactose. The lactase contents of organisms grown in lactose or galactose media were 20–400 times greater than those necessary to account for the rates of fermentation, while the rate of lactose hydrolysis by intact organisms (measured by the rate of lactose fermentation in the presence of 100 mg. of galactose-adapted *S. mandshuricus*) was little greater than the rate of fermentation. Table 7 gives the results of two experiments in which the lactase contents and the rate of lactose hydrolysis by intact organisms were compared for organisms grown in Roux bottles in basal media + lactose. It will also be seen that the rates of fermentation and hydrolysis of lactose by intact organisms showed little variation between pH 3.0 and 6.0, whereas lactase in disrupted organisms was inactive at pH 3.0. In this experiment the lactase content was determined on vacuum-dried *S. fragilis*, but CTAB-treated organisms behaved similarly.

With intact organisms the rate of hydrolysis of lactose slightly exceeds the rate of fermentation. To determine the nature of the sugars released into the

medium a thick (100 mg. dry wt./ml.) suspension of *S. fragilis* was allowed to ferment lactose for varying periods of time. Samples were centrifuged at 3000 g and the supernatant fluids run on paper chromatograms in ethyl acetate/pyridine/water. Before all the lactose had been utilized sugars with R_F values corresponding to lactose, glucose and galactose were detected. Visual comparison of the size and intensity of the glucose and galactose spots indicated that approximately equivalent amounts of these two sugars were present.

Galactose fermentation. *Saccharomyces fragilis*, grown in a medium containing an excess of glucose, was unable to ferment galactose. When the medium contained growth-limiting amounts of glucose the rate of galactose fermentation varied from zero to $Q_{CO_2}^N = 3.0$. When the yeast was grown in a medium containing lactose or galactose, the rate of galactose fermentation varied from 5 to 90 % of the rate of lactose fermentation (Table 6).

DISCUSSION

The formation of lactase and of invertase by *Saccharomyces fragilis* is similar in many respects. The two processes resemble each other in their response to glucose in the culture medium, the amount of enzyme formed being small in media containing glucose at concentrations exceeding 0.01 % (w/v) and increasing simultaneously at concentrations lower than this. Figs. 4 and 5 include several points for lactase content at a concentration of 0.001 % (w/v) glucose; the points with the lower activities probably correspond to a higher concentration of glucose in the medium than the points at higher activities, but the precise glucose concentrations were not ascertained since they were below the limit of sensitivity of the sugar determination procedure. Lactase formation is stimulated by growth of *S. fragilis* in the presence of the enzyme substrate lactose as sole source of carbon. The stimulation is greater at lower substrate concentrations, suggesting that there is some inhibition of lactase formation by higher concentrations of lactose (Table 2). Galactose produces a similar response (Table 3). Invertase formation shows a similar dependence on sucrose concentration (Davies, 1956). Lactase formation, like invertase formation, is independent of the growth rate of *S. fragilis* and of the concentration of ammonia-N and growth factors. In media containing excess glucose the lactase content of *S. fragilis* increases with rise in pH value and in this respect lactase formation is again similar to invertase formation. However, when the medium contains limiting amounts of glucose increasing the pH value from 3.4 to 7.7 has no significant effect on lactase formation, whereas invertase formation is minimal at pH 5.4 and its activity increases as the pH departs from this value, and in this respect the two enzymes differ. The work of Wilkes & Palmer (1932) and of Demis, Rothstein & Meier (1954) suggests that invertase is associated with the surface of the organism. The much greater dependence on pH value of the lactase activity of disrupted organisms as compared with intact organisms (Table 7) suggests that the lactase of *S. fragilis* is separated from the environment by an osmotic barrier. The existence of such a barrier might also account for the increase in lactase activity observed

on disruption of the organisms if it had a restricted permeability to lactose. The existence of 'permeability barriers' was invoked by Deere (1939) to account for similar increases in lactase activity when *Escherichia coli mutabile* was disrupted. On the other hand, Myrbäck & Vasseur (1943) suggested that at pH values where toluene-treated *Torula utilis* showed no lactase activity, fermentation of lactose by the intact organism must occur by some route not involving lactase. This explanation seems unlikely.

Table 7. *Comparison of lactose hydrolysis in intact Saccharomyces fragilis with lactase content of disrupted organisms*

S. fragilis was grown in Roux bottles in basal medium + lactose. In Expt. A the medium contained 2% (w/v) lactose initially, in Expt. B the initial lactose concentration was 1.0% (w/v). Washed suspensions were prepared and the lactase activity of vacuum-dried yeast, measured by carbon dioxide production from lactose in presence of *S. mandshuricus*, was compared with the rate of lactose hydrolysis by intact *S. fragilis* at different pH values. Results expressed as μ mole lactose hydrolysed or fermented/mg. dry wt. organisms/hr.

Expt. ...	A	B	B	B
pH value for enzymic tests	6.0	3.0	4.0	6.0
Lactase activity at the above pH values	46	0.05	0.15	82
Lactose hydrolysed by intact <i>S. fragilis</i> at the above pH values	5.5	4.2	4.8	5.3
Lactose fermented by intact <i>S. fragilis</i> at the above pH values	4.3	3.9	4.0	4.2

It is clear that growth of *Saccharomyces fragilis* in media containing lactose or galactose results in an increased formation of lactase and an increased rate of fermentation of lactose. If the rate of lactose utilization is partly controlled by the rate of migration across some barrier it is conceivable that growth in lactose or galactose media might modify the properties of the barrier in a way which would permit a more rapid passage of lactose. An increased rate of lactose fermentation might then result without a parallel increase in lactase content. There is no experimental evidence in support of this. The ratio of $Q_{CO_2}^{N_2}$ (lactose) to lactase content (expressed as a $Q_{CO_2}^{N_2}$ value) varied from 0.01 to 0.03 for those cases where lactose was fermented at a slower rate than glucose and where, therefore, the rate is probably not limited by the rate of overall glycolysis (Table 4). This variation is probably not significant. The rate of lactose fermentation appears to be roughly proportional to the lactase content under these conditions.

The existence of an osmotic barrier is not the only mechanism which can be invoked to account for the apparent inactivity of over 90% of the lactase in *Saccharomyces fragilis*. Other possibilities are: (a) the intracellular pH value is not optimal for lactase action; (b) the ionic composition within the cell may not be optimal for lactase action; (c) lactase may be in combination with a specific inhibitor within the organism; (d) the physical state of lactase within intact organisms may be different from that after their disruption. With respect to (a), the average pH value within cells of *S. cerevisiae* is around 5.8 (Conway & Downey, 1950) which is close to the pH value (6.0) at

which the lactase estimations were carried out in this work with *S. fragilis* and it seems unlikely that differences in hydrogen-ion concentration could account for the 10- to 30-fold difference in activity between intact and disrupted *S. fragilis*. It must be admitted, however, that we have no information regarding the local distribution of hydrogen ions within the organism; this also applies to the local distribution of potassium and sodium ions. The potassium-ion concentration of *S. fragilis* is of the order of $37.5 \mu\text{g./mg. dry wt.}$ corresponding to an average concentration of between 0.1 and 0.2M (R. Davies, unpublished results) which is sufficient for maximal lactase activity. This value for potassium-ion concentration agrees with the concentration worked out from the figure of 450 mg. potassium ion/100 g. baker's yeast found by Conway & Breen (1945). Lactase activity in the presence of 0.1M-potassium ion is depressed by the addition of sodium ion. However, 0.1M-sodium ion only halves the activity (R. Davies, unpublished results), and it seems unlikely that ionic imbalance can account for the difference in lactase activity of intact and disrupted *S. fragilis*. There is no evidence to support or refute possibilities (c) and (d), although Kaplan (1954, 1955) and Fraser & Kaplan (1955) suggested that differences in activity of yeast catalase as between intact and disrupted organisms might be due to differences in the physical state of the enzyme.

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Antibiotic Activity of Actinomycetes in Soil and their Controlling Effects on Root-rot of Wheat

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SUMMARY: A study has been made of conditions affecting the production of antibiotics in three soils by a number of unidentified *Streptomyces* spp. capable of inhibiting a variety of test organisms *in vitro*. In actinomycete-inoculated soils, antibiotic production was demonstrated only in sterile soils supplemented with a suitable organic source. The greatest accumulations of antibiotics were found in a neutral soil with added glucose (2.5 %) while under similar conditions, no antibiotics, or only traces were recovered from acid and alkaline soils. Antibiotics, however, could be recovered from inoculated acid soil, following neutralization and the addition of glucose. Fresh grass (3 %), clover (3 %) and soybean meal (2 %) were also suitable supplements for antibiotic production by the majority of the actinomycetes, though the amounts of antibiotics were considerably less than in glucose-treated soils.

In greenhouse experiments the assessment of root damage to wheat seedlings in sterile soil demonstrated that all the actinomycetes tested significantly reduced the degree of root-rot caused by *Helminthosporium sativum*. In the neutral and alkaline soils a relationship was evident between disease incidence and degree of antagonism exhibited by actinomycetes *in vitro*, suggesting that antibiotics were responsible. No such relationship was observed between disease control and the antibiotic-producing abilities of the actinomycetes in soil as determined by standard assay procedures.

Numerous investigations have been undertaken in attempts to distinguish between the various types of actinomycete antagonisms occurring in soil. High percentages of actinomycete soil isolates have been found to produce antibiotics *in vitro* and it is conceivable that these substances may be formed in soil and play an active role in microbial antagonism (Landerkin, Smith & Lochhead, 1950; Stevenson, 1953; Waksman, Horning, Welsch & Woodruff, 1942). Some evidence has already accumulated, showing that many actinomycetes can produce detectable quantities of antibiotics in sterile, supplemented soils (Gottlieb & Siminoff, 1952; Gregory, Allen, Riker & Peterson, 1952); but evidence of antibiotic production in natural soils is still lacking.

Attempts to control root diseases by inoculation of the infested soils with actinomycete isolates have met with considerable success. Cooper & Chilton (1947) tested various *Streptomyces* isolates and noted reduction of root-rot in sugar cane and corn plants infested with a *Pythium* sp. Other workers have noted similar disease control through the inoculation of antagonistic micro-organisms into soil (Lachance & Perrault, 1953; Slagg & Fellows, 1947). It is difficult to decide from such experiments whether the observed antagonism is due to antibiotic production or to some other form of antagonism.

Thaysen (1950) has emphasized the fact that although many micro-organisms may be antagonistic to others in culture this is not indicative of antibiotic activity in soil. In this respect it is significant that several workers have reported a relationship between the antibiotic activity of micro-organisms and their controlling effects on plant pathogenic fungi. Thus, Johnson (1952) was able to show that, with few exceptions, those actinomycete isolates which significantly reduced the root-rot of corn in soil were the most highly antagonistic on plates. Slagg & Fellows (1947) found a number of soil fungi antagonistic to *Ophiobolus* in pure culture and, of a selected group of these, most were found to be antagonistic to the fungus in soil. Anwar (1949) emphasizes the importance of antibiotic-producing organisms in soil and reports the control of root-rot of barley by these antagonistic types.

This paper reports on results of a preliminary investigation concerned with the antagonistic relationships between ten antibiotic-producing actinomycetes and the root-rot fungus *Helminthosporium sativum*.

METHODS

The actinomycetes. The ten actinomycetes used throughout were all unidentified *Streptomyces* spp. Those referred to as D22, E4, E63, F19, XX19 and Y27 were isolated by Landerkin *et al.* (1950) from the soils of northern Canada, while the actinomycetes AA24, AA27, AA30 and AA53 were isolated by Stevenson (1953) from soils of the Arno Atoll.

Determination of antibiotic activity. Antibiotic activity of the actinomycetes was determined by the cross-streak method against the following test organisms: *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, *Helminthosporium sativum*, *Fusarium culmorum* and *Streptomyces scabies*. The actinomycetes were streak-inoculated on plates of Conn's asparagine agar. After 5 days' incubation at 26°, the plates were flooded to the actinomycete growth with 3 ml. of a seeded agar medium suitable for the test organism. Plates were examined for antagonism after an appropriate incubation period and the inhibition zones measured.

Soils. Three contrasting soils were used, a highly acid (pH 5.0) sandy loam from Woburn, a medium loam soil (pH 6.9) from the Highfield plots of the Rothamsted Experimental Station, and a Cotswold limestone soil (pH 8.0) of the Sherborne soil series. These are referred to as the acid, neutral and alkaline soils respectively. All soils were sieved and brought to 50 % of their water-holding capacity. When sterilization was necessary the soils were autoclaved for 1 hr. at 15 lb./sq. in.

Inoculation of soils. Soil inocula were prepared in the following manner. Two ml. of a heavy aqueous suspension of actinomycete spores were added to test tubes containing 10 g. of sterile, washed, white sand. The sand + spore mixtures were then added to flasks containing 100 g. of the soil to be inoculated, and the whole shaken vigorously to ensure an even distribution of the spores throughout the soil.

Helminthosporium sativum soil inoculum was prepared in an identical manner.

Extraction of antibiotics from soil. Preliminary experiments demonstrated that the antibiotics produced by the unknown actinomycetes were ether soluble at the pH values of the soils concerned. After incubation of the actinomycete-inoculated soils at 25° for 14 days, the water content was estimated and appropriate quantities of the soils (5–25 g. dry weight) were extracted by vigorously shaking the soil for 10 min. with three successive equivalent volumes of ether. The bulked extracts were concentrated *in vacuo* and assayed for antibiotic activity.

Antibiotic assay. A paper-disk assay method was adopted for assay of the ether extracts of soil. Extracts were added to thin blotting-paper disks 15 mm. in diameter and dried at 37° for 30 min. The dried disks were then placed on plates seeded with *Staphylococcus aureus*, incubated for 24 hr. at 37° and the diameters of the zones of inhibition measured and recorded in mm. per given weight of soil.

Greenhouse studies. Three-inch clay pots with a capacity of 100 g. were sterilized and half filled with sterile soil. Actinomycete and *Helminthosporium sativum* soil-inocula were prepared separately in flasks containing sterile soil with 2% soybean meal. After incubation, flasks of the fungal and actinomycete inocula were combined, and 50 g. added to each pot. Check pots were also prepared with sterile and non-sterile soils containing the fungus only. The pots were then seeded with fourteen surface-sterilized wheat seeds and covered with a half inch layer of sterile soil.

Assessment of root-rot. The roots of wheat seedlings were carefully washed on removal from the pots and assessed for degree of root damage by a modification of the technique of McKinney (1923). The plants were separated into six classes according to degree of infection, and each plant given a numerical rating from 0 to 5. A final infection rating for all plants grown in a given treatment was arrived at by the following equation:

$$\frac{\text{sum of numerical ratings}}{\text{total no. of plants} \times 5} \times 100 = \text{infection rating (\%)}$$

RESULTS

Antibiotic spectra of the actinomycetes

The range of antibiotic activity of the ten actinomycetes against a variety of test organisms in Petri dish culture is given in Table 1. In view of later experiments designed to determine the antagonism of these actinomycetes towards *Helminthosporium sativum* in soil, particular attention is drawn to the varying degrees by which this fungus is inhibited *in vitro*.

Antibiotic production in soils

Antibiotic production by the actinomycetes in variously treated acid, neutral, and alkaline soils was also determined. The recovery of antibiotics from sterile, supplemented soils inoculated with the actinomycetes are given in Table 2.

In the neutral soil supplemented with 2.5 % glucose, appreciable concentrations of antibiotics were produced by the actinomycetes AA 24, AA 27, D 22, E 4, E 63 and F 19. In a similarly treated alkaline soil only small amounts of antibiotics were produced by four of these same actinomycetes. No antibiotics were detected in the supplemented acid soil.

Table 1. *Antagonistic properties of actinomycetes in vitro*

Actinomycetes	Test organisms					
	<i>S. aureus</i>	<i>B. subtilis</i>	<i>E. coli</i>	<i>S. scabies</i>	<i>H. sativum</i>	<i>F. culmorum</i>
	Zone widths in mm.; means of triplicate plates					
AA 30	10	9	0	12	4	2
AA 53	26	21	23	17	10	3
D 22	24	21	0	15	12	8
E 4	33	25	0	19	15	10
F 19	27	21	0	15	15	9
E 63	34	25	0	17	18	12
AA 27	12	4	7	3	20	15
AA 24	22	5	13	4	22	18
Y 27	18	13	0	10	24	20
XX 19	17	12	0	2	24	20

Further experiments were undertaken in which the glucose-supplemented acid and alkaline soils were neutralized by the addition of calcium hydroxide and hydrochloric acid respectively. Under these modified conditions actinomycete growth was evident in the acid soil and antibiotics were detected. Inasmuch as no antibiotics were detected in this soil previously, it is suggested that the low acid tolerance of the actinomycetes limited their activity in the soil. Neutralization of the alkaline soil did not improve antibiotic production by the actinomycetes.

Antibiotic production was also demonstrated in sterile, neutral and alkaline soils supplemented with such natural organic materials as fresh grass (3 %) and clover cuttings (3 %). Table 2 illustrates that the amounts of antibiotics detected were considerably smaller than those produced by some of the actinomycetes in the glucose-supplemented soils, but indicates that the majority of these organisms were capable of producing some antibiotic with these supplements.

The production of antibiotics in sterile soils supplemented with soybean meal was also determined. Soybean meal is the high protein, low carbohydrate remnants of soybeans after oil extraction: it was supplied by the British Oil Cake Mills Ltd., Erith, Kent. The amounts of antibiotic extracted under these conditions were of the same order as those found in the grass and clover-supplemented soils. The actinomycetes again failed to produce detectable quantities of antibiotics in the acid soil.

Effects of actinomycetes on the incidence of root-rot of wheat

The following studies were undertaken to determine the potential antagonism of the actinomycetes towards *Helminthosporium sativum* in the acid, neutral, and alkaline soils. In these experiments the degree of activity of the

Table 2. Antibiotic production by actinomycetes in sterile, supplemented soils

Inhibition of <i>Staphylococcus aureus</i> by soil extracts. Zones of inhibition in mm.: *, activity/g. soil; †, activity/25 g. soil.															
Actino- mycetes	Glucose (2.5 %) supplemented soils*			Neutralized glucose (2.5 %) supplemented soils*		Grass (3 %) supplemented soils†		Clover (3 %) supplemented soils†		Soybean (2 %) supplemented soils†					
	Acid	Neutral	Alkaline	Acid	Alkaline	Neutral	Alkaline	Neutral	Alkaline	Acid	Neutral	Alkaline			
AA30	0	0	0	0	0	0	0	0	0	0	0	0			
AA53	0	0	0	0	0	0	0	0	0	0	0	0			
D22	0	19.0	0	17.0	0	19.0	18.5	18.5	18.5	0	19.0	0			
E4	0	25.0	0	19.0	0	22.0	20.0	21.0	21.0	0	17.5	0			
F19	0	18.5	0	15.0	0	21.0	19.0	21.0	21.0	0	18.5	0			
E63	0	35.0	16.5	18.0	0	17.5	17.5	18.5	17.5	0	0	20.0			
AA27	0	18.5	0	0	0	17.5	16.0	18.5	16.0	0	22.0	21.0			
AA24	0	18.0	0	0	0	16.0	0	17.5	16.0	0	20.0	19.0			
Y27	0	0	0	0	0	0	0	16.0	16.0	0	19.0	20.0			
XX19	0	0	0	0	0	19.0	0	18.5	0	0	0	17.5			

actinomycetes was measured in terms of their ability to reduce root-rot of wheat in soils inoculated with the fungus.

Twelve treatments were prepared in triplicate for each soil. Ten treatments consisted of a mixture of *Helminthosporium sativum* in combination with one of the ten actinomycetes in sterile soil. A treatment of *H. sativum* in non-sterile soil and control treatment of sterile soil plus *H. sativum* were also prepared.

At the end of 60 days of growth, seedlings were removed from the pots, the degree of root-lesioning assessed, and the infection ratings calculated for each pot, treatment, and soil. To aid in the comparison of actinomycete effects between the different soils, the infection ratings were converted on the basis of the appropriate controls (sterile soil plus *Helminthosporium sativum*) being 100 % infected. The average infection ratings for treatments and soils are given in Table 3.

Table 3. *Average infection ratings of wheat seedlings (%)*

	Soils			<i>In vitro</i> inhibition*
	Acid	Neutral	Alkaline	
Sterile soil plus <i>H. sativum</i>	100.0	100.0	100.0	—
Non-sterile soil plus <i>H. sativum</i>	47.9	31.0	29.0	—
AA 30	62.6	76.5	83.5	4
AA 53	62.8	83.8	82.8	10
D 22	77.4	64.0	87.6	12
E 4	74.4	69.9	82.5	15
F 19	59.4	73.2	81.0	15
E 63	73.1	60.7	74.7	18
AA 27	58.9	68.8	62.9	20
AA 24	62.8	47.9	61.0	22
Y 27	81.5	49.2	83.8	24
XX 19	68.2	35.5	82.9	24
L.S.D.†	14.5	11.9	8.7	—

* From Table 1. Inhibition zones in mm.

† Least significant differences (1 %).

Table 4. *Analysis of variance of wheat infection ratings*

Source	D.F.	Mean squares
Treatments (T)	11	744.9*
Soils (S)	2	779.3*
T × S	22	146.8*

* Significant at 1 %.

The analysis of variance of the data in Table 4 indicates that the actinomycete treatments and soils have both influenced the degree of root-rot of wheat. The least significant differences (Table 3) illustrate that all treatments have significantly reduced disease incidence in relation to the control treatments while the treatment-soil interaction of the analysis (Table 4) shows that the controlling effect of the individual treatments varies with the different soils.

Inasmuch as all actinomycete treatments have reduced root-rot of wheat, the degree of control was compared with the ability of the individual actinomycetes to produce antibiotics in soil as well as with their *in vitro* inhibition of *Helminthosporium sativum*. Of the ten actinomycetes studied, D22, E4, E63 and F19 were the most consistent producers of antibiotics in the sterile-

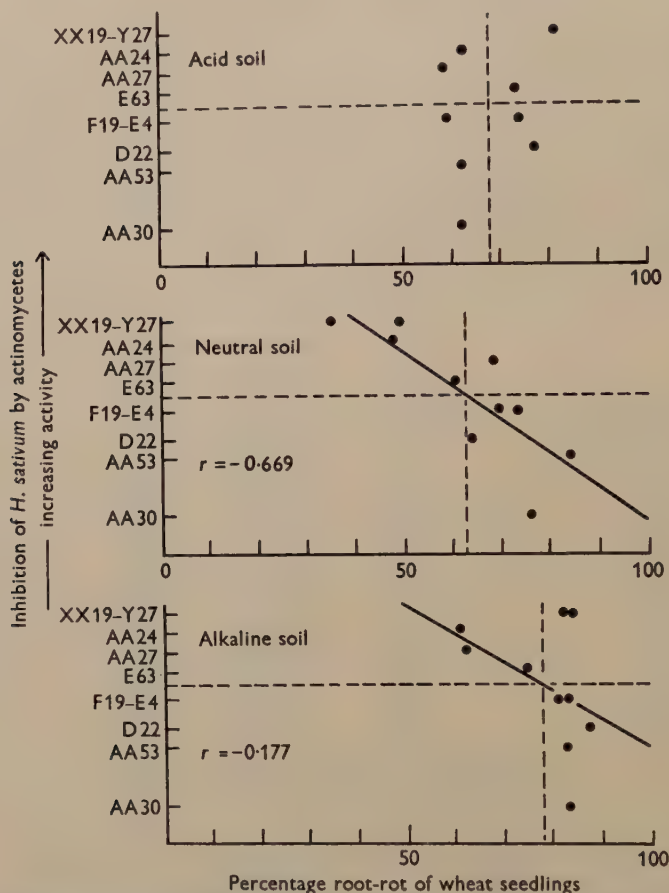


Fig. 1. Scatter diagrams indicating the relationship between the *in vitro* and *in soil* antagonism of ten actinomycetes towards *Helminthosporium sativum*.

supplemented soils (Table 2). The remaining organisms varied in their ability to produce these substances, though rarely did the amounts detected by soil extraction exceed those produced by the aforementioned actinomycetes.

Table 3 indicates that the controlling effects of the organisms D22, E4, E63 and F19 generally fall between those actinomycetes bringing about the greatest reduction in root-rot and those with the least effect. Further comparison shows that the actinomycetes most antagonistic to *Helminthosporium sativum* *in vitro* (Table 1) have the greatest controlling effect on the fungus in the neutral soil. This relationship is illustrated in Fig. 1, where the infection ratings

for the actinomycete treatments are plotted against the inhibition zones of *H. sativum* produced by the actinomycetes on plates (Table 1). When the data are plotted in this manner a pattern is seen in the neutral and alkaline soils. A significant coefficient of correlation was obtained only in the case of the neutral soil data. The scatter of points for the alkaline soil shows a definite tendency to follow the same pattern, but in this soil strains XX19 and Y27 are poor antagonists. The lack of such a pattern in the acid soil is attributed to the low acid tolerance of the actinomycetes.

DISCUSSION

In the investigations described, evidence has been produced indicating a relationship between the antagonistic effects displayed by a number of actinomycetes in sterile soil and their antibiotic activity *in vitro*. This evidence was illustrated by an apparent correlation between the control of root-rot of wheat in sterile soil and the inhibition of the causal fungus by the actinomycetes in Petri dish culture. No relationship was noted between disease control and the ability of these actinomycetes to produce antibiotics in soils as demonstrated by extraction techniques.

Although the ecological significance of antibiotics in soil has often been criticized on similar failures to demonstrate antibiotic production in soil, the possibility remains that these substances are present and active in micro-environments, where a relatively high concentration of antibiotic may be attained in the immediate vicinity of the antagonists, even though the total quantity of antibiotic per gram of soil may be far too small to be detected.

The frequent failure to relate the activity of antagonists *in vitro* and in soil may thus be due in part to the inadequacy of existing techniques for the detection of antibiotics in soil.

In a preliminary note (Stevenson, 1954) the author has suggested a new and more sensitive technique for demonstration of antibiotic activity in soil and a fuller account of this method will be presented in a later paper.

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Effects of Clupein and of its Degradation Products on a Rhizobium Bacteriophage, on its Host Bacterium and on the Interaction between the two

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SUMMARY: Clupein at 0.02-0.05 % in the liquid nutrient medium used to cultivate nodule bacteria, rapidly killed the bacteria and slowly inactivated a bacteriophage that attacked them. When added to a bacterial culture in liquid medium before adding phage, clupein prevented phage and bacteria from combining; when added after the two had combined, clupein interrupted further stages of phage-host interaction. Clupein at 0.0016 % acted bacteriostatically and slowed phage multiplication but did not stop it.

Trypsin and chymotrypsin hydrolyse clupein, trypsin breaking about twice as many peptide bonds as chymotrypsin. At a concentration corresponding to 0.02-0.05 % clupein, the peptides produced by chymotrypsin acted bacteriostatically in the liquid nutrient medium; the peptides inactivated phage much more slowly than did intact clupein, and they inhibited phage multiplication by interfering with the combination between phage and host. When added after phage and bacteria had combined, the peptides did not interfere with further stages of phage-host interaction. The smaller peptides produced by trypsin had no effect on host bacteria, phage, or phage/host interaction.

Phage preparations partially inactivated by clupein had their activity partially restored by incubation with trypsin or chymotrypsin.

Clupein, but none of its hydrolytic products, made phage with much non-phage material sedimentable by slow-speed centrifugation.

Bawden & Pirie (1937) found that tobacco mosaic virus was precipitated by clupein. Kleczkowski (1946) showed that, like many other proteins, the virus was precipitated only within the pH range in which it was charged oppositely to clupein. Pairs of some other proteins also precipitate each other in such conditions, presumably because electrostatic attraction leads to combination and mutual discharge.

Some protamines, particularly clupein, are highly toxic to animals when injected intravenously (Thompson, 1900). Clupein also inhibits the infectivity of tobacco mosaic virus when present in the inoculum, but it does not destroy the virus (Kleczkowski, 1946). The inhibitory effect of clupein can be diluted out, and virus with its original infectivity can be re-isolated from mixtures with clupein (Bawden & Pirie, 1937). Protamines do not seem to destroy some animal viruses, and have been used to fractionate crude virus preparations, either by precipitating viruses and leaving behind some other materials, or vice versa (Chambers & Henle, 1941; Warren, Weil, Russ & Jeffries, 1949, 1950). The work described in this paper was started to see whether clupein could be used to fractionate preparations of a rhizobium bacteriophage; it developed into a study of effects of clupein and of some of its degradation products on the phage, the host bacterium and their interaction.

METHODS

The phage for a strain of nodule bacteria (*Rhizobium leguminosarum*, strain 317), the media and the method of poured plates for obtaining plaques were the same as those used previously (Kleczkowski & Kleczkowski, 1954*a*, *b*). Bacterial cultures in liquid medium lysed by the phage and passed through a Chamberland L3 filter are referred to as 'stock cultures' of the phage.

Clupein sulphate was obtained from The British Drug Houses Ltd. Unless otherwise stated, the pH value of its solutions was adjusted to 7.

Preparations of crystallized trypsin and chymotrypsin were obtained from beef pancreas as described by Northrop, Kunitz & Herriot (1948). Chymotrypsinogen was crystallized eight times, and converted into chymotrypsin, which was crystallized twice. Trypsin was crystallized once. Trypsinogen was not crystallized.

Concentrations of active phage in various fluids are given as numbers of plaques/ml. and those of viable bacteria as numbers of colonies/ml. The fluids for phage assay were appropriately diluted in a 24 hr. liquid culture of the host bacteria, and those for bacterial assay in the sterile liquid medium. The diluted fluids were then plated by mixing 1 ml. of each with 9 ml. of melted 0.7% agar medium cooled to 42° and pouring into Petri dishes of 8 cm. diameter. Plaques were counted after 2 days of incubation at 25°; bacterial colonies after 5 days of incubation.

The work was complicated by the presence in the nutrient media of materials that reacted with clupein to form precipitates which settled under gravity or an opalescence that could be removed by centrifugation for 20 min. at *c.* 10,000 r.p.m. Most of the experiments were made with the strain 317 of nodule bacteria grown in the medium introduced by Demolon & Dunez (1935) to isolate rhizobium bacteriophages from the soil. In addition to inorganic salts, the medium contained an extract from lucerne roots. (The medium was modified by using half the amount of the extract, and it was clarified by filtration.) The medium became opalescent on adding clupein and all the material responsible for this opalescence could be removed by adding 0.1 mg. clupein sulphate/ml. of medium, and then centrifuging.

The other medium used was a nutrient broth used with *Escherichia coli* and with *Staphylococcus aureus*, which were studied only for the bactericidal effect of clupein. Additions of clupein sulphate to this medium produced an abundant precipitate, and at least 0.6 mg. clupein sulphate/ml. of the medium were needed to remove all the material which precipitated with clupein. This material protected the bacteria in broth from any detectable effect by 0.05% clupein sulphate, and tests for bactericidal effects were therefore made with bacteria suspended in physiological saline. The nodule bacteria were not protected in the nutrient medium of Demolon & Dunez from the effects of as little as 0.0015% clupein sulphate. All the experiments with the nodule bacteria and with the bacteriophage were, therefore, done in this medium, and it has to be borne in mind that some proportion of the added clupein was probably bound by materials present in the medium, so that only part was responsible for the effects described below.

RESULTS

Analysis of clupein and hydrolysis by trypsin and chymotrypsin

The amino acids of clupein were identified by paper chromatography. Clupein sulphate (50 mg.) was dissolved in 2.5 ml. of 6N-HCl and heated in a sealed tube for 24 hr. at 100°. Hydrochloric acid was removed by heating the solution at 100° in vacuum, dissolving the dry residue in water and evaporating the water at 100° in vacuum three times. The final residue was dissolved in 5 ml. H₂O and 0.025 ml. of the solution was placed on a Whatman no. 1 filter paper for chromatographic analysis. The chromatogram was developed with phenol + NH₃ in one direction, with 'collidine' in the other and stained with ninhydrin. In addition to the spots corresponding with the five amino acids known to occur in clupein (arginine, valine, proline, serine and alanine (Kossel & Dakin, 1904; Waldschmidt-Leitz, Ziegler, Schöffner & Weil, 1931)), three weaker spots were obtained corresponding to threonine, glycine and leucine or isoleucine. Colorimetric determination of arginine in the clupein hydrolysate by Weber's modification of Sakaguchi's method (Weber, 1930) showed that the arginine content of clupein was about 80 %, which agrees with previous statements that clupein contains twice as many residues of arginine as of all other amino acids taken together.

Hydrolysis of clupein by trypsin and chymotrypsin was examined by adding 0.5 ml. of a 0.5 % solution of trypsin or chymotrypsin (or of a mixture of equal volumes of the two) to 4.5 ml. of a 2 % solution of clupein sulphate at pH 7.2, and incubating for 48 hr. at 37°. In controls water was substituted for enzyme solutions or for the solution of clupein sulphate. After incubation 0.01 ml. of each solution was placed on filter-paper for one-dimensional chromatography and the remainder was used for formol-titration. This was done by adjusting the pH value (measured with a glass electrode) to 7.0 in all the solutions, adding 2.5 ml. of a 20 % solution of formaldehyde at pH 7.0, and titrating back to pH 7.0 with 0.02N-NaOH. The amounts of 0.02N-NaOH used were: none with control solutions containing the enzymes alone; 0.8 ml. with the control containing clupein sulphate alone; 5.7, 3.7 and 5.7 ml. respectively with solutions of clupein sulphate which had been incubated with trypsin, with chymotrypsin, and with the two enzymes together. Thus chymotrypsin split only about a half of the peptide bonds in clupein that were split by trypsin, which agrees with previous observations made by other methods (Waldschmidt-Leitz & Kofranyi, 1935).

The chromatogram of the clupein hydrolysates and of the controls was developed for 48 hr. with a mixture of 10 vol. water-saturated *n*-butanol + 1 vol. glacial acetic acid, with enough water added to the mixture just to reach saturation, and stained with ninhydrin. Pl. 1, fig. 1, shows only a region of the upper part of the chromatogram; the lower part contained no spots. No spots appeared anywhere when the enzymes alone were run. Pl. 1, fig. 1, also shows the spot produced by 0.01 ml. 0.1 % arginine, and the positions of other spots are given below in terms of R_{arg} values in relation to the position of the arginine spot. All the spots were purple, though varying intensity.

Unhydrolysed clupein gave a very faintly stained spot with $R_{\text{arg}}=0$. Clupein hydrolysed by chymotrypsin produced two intensely stained overlapping spots with R_{arg} values of 0 and *c.* 0.15, and a few very faintly stained spots. Clupein hydrolysed by trypsin produced two still more intensely stained spots with R_{arg} values of *c.* 0.15 and 1.0, and a few faintly stained spots. The most intensely stained spot with $R_{\text{arg}}=1.0$ was probably produced by free arginine split by trypsin from clupein. A colorimetric determination of arginine by the modified Sakaguchi's method in a water extract from a section of another non-stained chromatogram showed that about one-fifth of the total arginine of trypsin-hydrolysed clupein was in the area corresponding with the free arginine spot. With the solvent used arginine was the slowest of all the amino acids contained in clupein, and as there were no spots below that of arginine, this was the only free amino acid split from clupein by trypsin in a detectable amount. The spots above the arginine spot were, therefore, produced by peptides, most of which corresponded with the R_{arg} value of *c.* 0.15. Chymotrypsin did not split off any free amino acids in detectable amounts and broke clupein into peptides, most of which corresponded with the R_{arg} values of 0 and 0.15. The chromatogram of clupein hydrolysed by trypsin and chymotrypsin together did not differ from that of clupein hydrolysed by trypsin alone.

According to Bergmann (1942) chymotrypsin requires for its activity the presence in its substrates of a tyrosine or phenylalanine residue. As clupein has no such residues and is hydrolysed by chymotrypsin, Bergmann's definition of requirements of chymotrypsin is too narrow. The fact that trypsin splits all the bonds in clupein which can be split by chymotrypsin plus some that are not, also conflicts with Bergmann's classification of proteolytic enzymes, according to which trypsin differs from chymotrypsin in the ability to split different peptide bonds.

Effect of clupein on numbers of bacterial colonies and on numbers of plaques

To find the maximum concentration of clupein which did not affect the results of the assays for viable bacteria and active phage in the agar medium, 0.5 ml. vol. of solutions of clupein sulphate at different concentrations, or 0.5 ml. of water, were added to a series of tubes each containing 9 ml. of melted agar medium cooled to 42°. When effects on numbers of bacterial colonies were to be tested, 0.5 ml. of a 24 hr. liquid bacterial culture diluted $1/10^5$ was then added to each tube. When effects on numbers of plaques were to be tested, the addition was 0.5 ml. of undiluted liquid bacterial culture containing phage stock culture diluted $1/10^7$. The contents of the tubes were then poured into Petri dishes and incubated at 25°. Plaques were counted after 2 days of incubation and bacterial colonies after 5 days. (Addition of sufficiently high concentrations of clupein to the agar medium caused flocculation, but the floccules dispersed on stirring and solidified agar plates with clupein did not differ in appearance from control plates without clupein.)

Table 1 shows that the transition from complete suppression of plaques

and colonies to full production of both was rather sharp when the concentration of clupein sulphate decreased from 0.05 to 0.025 %. Therefore, when assaying concentrations of viable bacteria or of active phage, care was taken not to introduce clupein sulphate in amounts that would exceed 0.025 % in the agar medium.

Table 1. *Effect of the presence of clupein in agar medium on numbers of bacterial colonies and on numbers of plaques*

Concentrations of clupein sulphate in agar medium (%)	Total numbers of bacterial colonies on four plates	Total numbers of plaques on four plates
0.05	0	0
0.025	448	247
0.0125	456	295
0.00625	485	281
0 (control)	444	275

Effects of clupein on host bacterium, on phage and on phage/host interaction in liquid medium

To test the effect of clupein on the bacteria in the liquid medium, 0.5 ml. of solutions of clupein sulphate at different concentrations were added to a series of tubes each containing 4.5 ml. of a 24 hr. bacterial culture in liquid medium. The mixtures were incubated at 25°, and samples withdrawn at intervals to assay viable bacteria. Table 2 shows that at and above a concentration

Table 2. *Effect of clupein on host bacteria in the liquid medium*

Concentrations of clupein sulphate in the medium (%)	Concentrations of viable bacteria (numbers of colonies/ml. culture) after a period of incubation of	
	1 hr.	24 hr.
0.05	5	0
0.016	170	3
0.005	75×10^3	2×10^3
0.0016	11×10^6	11×10^6
0.0005	43×10^6	54×10^6
0 (control)	41×10^6	79×10^6

The initial concentration of viable bacteria was about the same as in the control after 1 hr. of incubation.

of 0.005 % clupein killed the bacteria at a higher rate with increasing concentration. At 0.0016% clupein acted bacteriostatically, and at 0.0005% it still slightly inhibited bacterial growth. The bacteria seem to be killed by much smaller concentrations of clupein in liquid than in agar medium (see Table 1). The difference may be due to more clupein combining with constituents of the agar medium than with those of the liquid medium, or to its relatively slow diffusion through agar.

To determine whether clupein had any more general bactericidal action, two other bacteria, a strain of *Escherichia coli* and a strain of *Staphylococcus aureus* isolated from healthy human skin, were tested. When nutrient broth in which these organisms were grown contained 0.05 % clupein sulphate, both bacteria grew at their normal rates. However, addition of clupein sulphate to this medium immediately produced a large precipitate; it seemed likely, therefore, that the bacteria had been protected from clupein because most of the latter had combined with some constituents of the medium. Indeed, when the bacteria were suspended in physiological saline and clupein sulphate was added to 0.05 %, all the *Escherichia coli* organisms and most of the staphylococci were killed during 3 hr. of incubation at 25°, forming no colonies when plated on agar medium.

Table 3. *Effect of clupein on the bacteriophage*

Concentrations of clupein sulphate in the medium (%)	Concentrations of active phage (numbers of plaques/ml.) ($\times 10^{-7}$) after	
	24 hr.	48 hr.
0.05	3	0.5
0.025	6	1.5
0.0125	17	9
0.00625	30	28
0.003125	52	41
0 (control)	66	63

The initial phage concentration was the same as in the control.

The effect of clupein on the phage was tested by adding 0.5 ml. of solutions of clupein sulphate at different concentrations to a series of tubes each containing 4.5 ml. of undiluted phage stock culture. The mixtures were incubated at 25° and samples were taken at intervals for assaying phage. Table 3 shows that when the concentration of clupein sulphate was 0.003 % or higher, the phage activity of the preparation decreased with increasing concentration of clupein, though very slowly by comparison with the rates at which the bacteria were killed at the same concentrations of clupein (see Table 2).

The effect of clupein on phage multiplication was tested by adding 0.5 ml. of solutions of clupein sulphate at different concentrations to a series of tubes each containing 4 ml. of a 24 hr. bacterial culture in liquid medium and then adding to each tube 0.5 ml. of a phage stock culture at a dilution of $1/10^5$. The mixtures were incubated at 25° and samples were taken at intervals to assay phage. Comparison of the results in Table 4 with those in Table 2 shows that phage multiplication remained unaltered only at a concentration of clupein at which bacterial growth also remained almost unaffected (0.0005 %). At a concentration at which clupein was bacteriostatic (0.0016 %) phage multiplied but more slowly than in the absence of clupein. Finally, at bactericidal concentrations of clupein (0.005 % and above) phage activity decreased in a manner similar to that of phage alone exposed to clupein.

To determine whether clupein affected only some initial stage of phage/host interaction, such as combination between phage and host, or whether it also interfered with further stages of the interaction, bactericidal amounts of clupein sulphate were added to the bacterial cultures which had been incubated with phage for 30 min. at 25°, so that most of the phage had already combined with bacteria. The mixtures were further incubated for 3·5 hr., by which time infected organisms would normally have lysed and the concentration of phage in the medium would have increased about 100-fold. Not only was there no

Table 4. *Effect of clupein on phage multiplication in a liquid bacterial culture*

Concentrations of clupein sulphate in the medium (%)	Concentrations of active phage (numbers of plaques/ml.) after	
	4 hr.	24 hr.
0·05	220	40
0·016	440	140
0·005	550	410
0·0016	10,000	83×10^8
0·0005	105,000	34×10^8
0 (control)	96,000	31×10^8

The initial phage concentration was 750 plaques/ml.

increase in the concentration of phage but there was a decrease to less than one-tenth of the concentration obtained when clupein was added to the bacterial culture before adding phage. Thus the normal course of development of phage that had combined with bacteria before adding clupein was arrested and the phage became inactive. It still remains unknown whether phage multiplication inside bacterial cells was immediately arrested by clupein or whether phage continued to multiply but was not released because the bacteria did not lyse. It is clear, however, that the presence of clupein in bactericidal concentration prevented phage and bacteria from combining and also interrupted the normal course of phage development, if this had already started in the absence of clupein.

Influence of sodium chloride on the effects of clupein

Various effects of clupein on the phage, on the host bacterium and on their interaction might be results of combination of phage and of bacteria with clupein, caused by electrostatic attraction. Such combination is to some extent prevented or reversed by inorganic salts such as NaCl (Kleczkowski, 1946). It seemed possible, therefore, that some of the effects of clupein might be prevented or reversed by 2 % NaCl, which is sufficient to clarify or to prevent the appearance of opalescence caused by the presence of clupein in the nutrient medium. However, the salt itself had bactericidal effect on the host bacteria, decreasing the concentration of viable organisms to 12 % of the original during 24 hr. of incubation at 25°, and it completely inhibited phage multiplication in bacterial culture in liquid medium. The much more powerful bactericidal

effect of 0.05 % clupein sulphate remained unaffected by the salt. On the other hand, the phage itself was not affected by the salt. In the presence of 2 % NaCl, phage activity of a stock culture was only halved by 0.05 % clupein sulphate during 24 hr. of incubation at 25°, whereas it was decreased to 10 % of the original in the absence of the salt. Phage inactivated by clupein was not reactivated by addition of salt.

Effects of degradation products of clupein

Tests similar to those made with clupein were also made with clupein hydrolysed into its constituent amino acids by HCl. At a concentration equivalent to 0.05 % of the original clupein sulphate the hydrolysate had no effect on the host bacteria, on the phage or on phage/host interaction. The constituent amino acids of clupein taken singly at 0.05 % had also no effect. Similarly, hydrolysates produced by incubating clupein with trypsin had no effect on phage, but the chymotrypsin hydrolysate decreased phage activity of a stock culture to 75 % of the original during 24 hr. of incubation at 25°. Phage activity was decreased to 12 % in a simultaneous experiment by 0.05 % intact clupein sulphate.

A test was then made to see whether activity of a phage culture, decreased to 12 % of its original activity by incubation with intact clupein, could be restored by subsequent incubation with trypsin or chymotrypsin. One-tenth volume of a 0.5 % solution of trypsin or chymotrypsin was added to the culture which had been incubated with intact clupein, and the culture was again incubated for 3 hr. at 37°. This resulted in a restoration of phage activity to 40 % of the original where trypsin was added, and to 20 % where chymotrypsin was added. It is possible, therefore, that the inactivation of phage by clupein was at least partially due merely to combination between phage and clupein, perhaps followed by aggregation. This would be reversed by trypsin and, to a smaller extent, by chymotrypsin. However, the part of the inactivating effect that was not reversed by the enzymes might be due to structural alterations in phage particles.

The investigation of the effect of clupein hydrolysed by trypsin and chymotrypsin on the phage/host interaction is complicated by the fact that these enzymes themselves affect the interaction. Chymotrypsin has no effect on the phage or the host bacteria separately but affects the interaction between the two, inactivating phage particles immediately after they have combined with bacteria (Kleczkowski & Kleczkowski, 1954*a, b*). Trypsin behaves similarly and has about the same lowest effective concentration, namely about 0.001 %. It was, therefore, necessary not to exceed this concentration when testing for effects of hydrolysed clupein on phage/host interaction.

The following five mixtures, all at pH 7.2, were incubated for 24 hr. at 37°:

- (A) 0.9 ml. 5.5 % clupein sulphate + 0.1 ml. water;
- (B) 0.9 ml. 5.5 % clupein sulphate + 0.1 ml. 0.5 % trypsin;
- (C) 0.9 ml. 5.5 % clupein sulphate + 0.1 ml. 0.5 % chymotrypsin;
- (D) 0.9 ml. water + 0.1 ml. 0.5 % trypsin;
- (E) 0.9 ml. water + 0.1 ml. 0.5 % chymotrypsin.

After the incubation all the fluids were diluted 1/25 in water and 0.5 ml. of each diluted fluid, or 0.5 ml. water, was added to 4 ml. of a 24 hr. bacterial culture in liquid medium, followed either by 0.5 ml. of a phage stock culture at a dilution of 1/10⁵ when the effect on phage multiplication was tested, or by 0.5 ml. of the sterile liquid medium when the effect on the bacteria was tested. Thus, the final concentration of clupein sulphate in the medium was 0.02 %, enough for unhydrolysed clupein to have a powerful bactericidal effect and to prevent phage multiplication completely, whereas the concentration of the enzymes was 0.0002 %, i.e. below the limiting effective concentration for inhibition of phage/host interaction. The mixtures were incubated at 25° and

Table 5. *Effects of enzymic degradation products of clupein on multiplication of phage and of host bacteria*

Tested materials	Concentrations of active phage (numbers of plaques/ml.) after		Concentrations of viable bacteria (numbers of colonies/ml.) after	
	4 hr.	24 hr.	3 hr.	24 hr.
Intact clupein (mixture A)	910	210	210	0
Clupein hydrolysed by trypsin (mixture B)	14×10^4	34×10^8	6×10^7	10×10^7
Clupein hydrolysed by chymotrypsin (mixture C)	970	85×10^8	5×10^7	6×10^7
Trypsin alone (mixture D)	13×10^4	35×10^8	6×10^7	11×10^7
Chymotrypsin alone (mixture E)	15×10^4	31×10^8	5×10^7	11×10^7
Control (water)	13×10^4	33×10^8	6×10^7	10×10^7

The initial concentration of phage was 1320 plaques/ml., and that of viable bacteria was about the same as in the control after 3 hr. incubation.

samples were taken at intervals for assays. Table 5 shows that clupein hydrolysed by trypsin had no effect either on phage multiplication or on bacterial multiplication. Clupein hydrolysed by chymotrypsin had no bactericidal effect, but it had a bacteriostatic effect and it inhibited phage multiplication, completely during the first 4 hr. and considerably during the following 20 hr.

The chymotrypsin hydrolysate of clupein interfered with combination between phage and host. This was demonstrated by adding the hydrolysate to a bacterial culture, then adding phage, incubating the mixture for 30 min. at 25°, sedimenting the bacteria by centrifugation for 20 min. at 10,000 r.p.m. and assaying phage in the supernatant fluid and in the resuspended sediment. Most of the phage remained in the supernatant fluid, whereas in a control experiment, in which water was substituted for the hydrolysate, most of the phage was in the sediment.

To examine whether further stages of phage/host interaction were interfered with, the hydrolysate was added to a phage+bacteria mixture which had previously been incubated for 30 min. so that most phage particles had already combined with bacteria; phage was assayed after a further 3.5 hr. of incubation, by which time normally all the infected bacteria would have lysed. Almost as much phage was detected as in a control experiment without the

hydrolysate, i.e. about a 100-fold increase in phage concentration had occurred. Thus the cycle of phage/host interaction, which had already started, proceeded to completion in the presence of the hydrolysate.

Thus, none of the constituent amino acids of clupein or the peptides produced by trypsin showed any of the effects of clupein on phage or bacteria, whereas the larger peptides produced by chymotrypsin did show some effect. The size of molecules may be important, but the effects might also be functions of some configurations that were destroyed when some peptide bonds were broken.

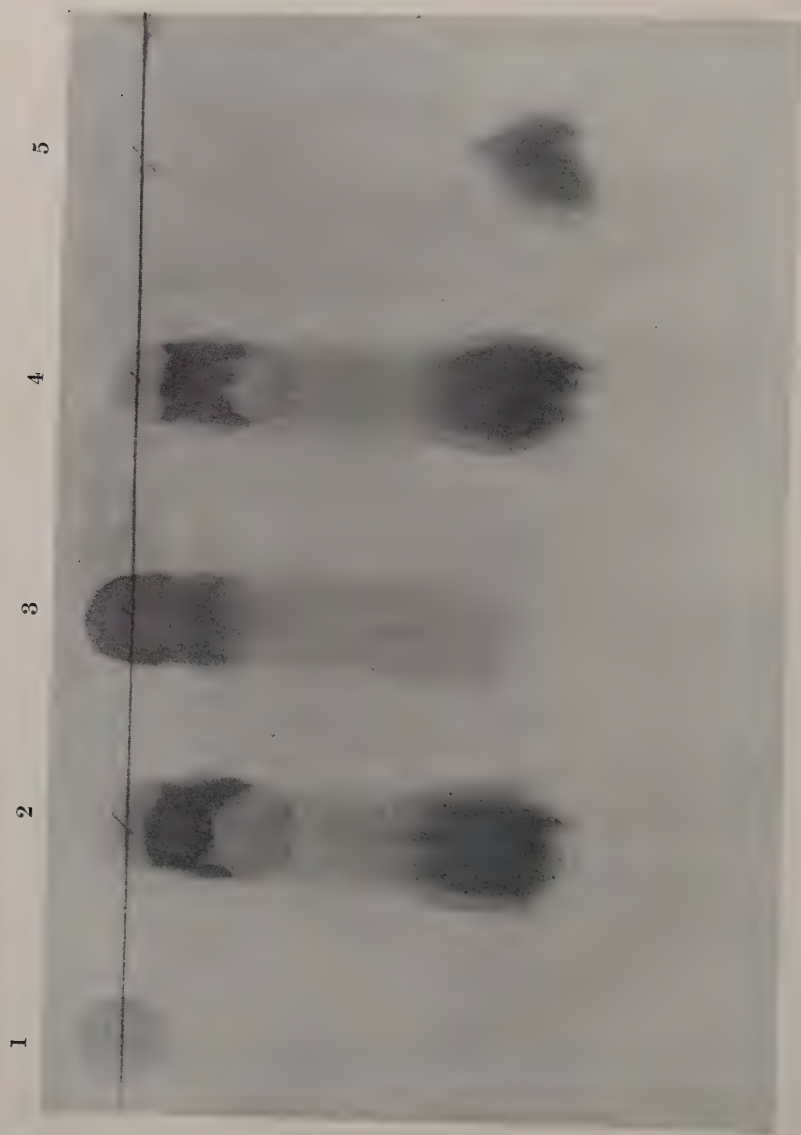
Sedimentation and resuspension of phage + clupein complex

All centrifugations referred to below were done in an angle centrifuge at *c.* 10,000 r.p.m. for 20 min. (about 6000*g* at the middle of the tube). Centrifugation of a phage stock culture did not sediment phage appreciably, but most of the phage sedimented from cultures to which 0.02–0.05 % clupein sulphate had been added. The clupein sulphate made the culture opalescent, and centrifugation separated a sediment and left a clear supernatant fluid. By resuspending the sediment various amounts of phage activity were recovered, the amounts depending on the kind of suspending medium used and on other factors (e.g. concentration of clupein; length of time of exposure of phage to clupein). Usually, less than 10 % of phage activity was recovered when the sediment was suspended in water, up to 50 % in 2 % NaCl and up to 80 % in the nutrient medium. Up to 80 % was also regained when the sediment was suspended in a 0.1 % water solution of trypsin and incubated for a few hours at 37° at about pH 7.

The presence of 2 % NaCl in a phage culture or hydrolysis of clupein by trypsin or chymotrypsin before adding it to the culture, prevented the culture from becoming opalescent and phage from becoming sedimentable by centrifugation.

Most of the material sedimented from a phage culture is not phage, for about as much sediment is obtained from the sterile medium. The quantity of sediment was decreased by incubating the culture with trypsin or chymotrypsin before adding clupein. The opalescence produced by adding clupein to such cultures decreased on incubation and disappeared after *c.* 1 hr. at room temperature. The amount of phage sedimentable by centrifugation also decreased with increasing length of time between the addition of clupein and centrifugation of the fluid; no phage was sedimented when the opalescence had disappeared.

Phage can be concentrated by suspending the sediment in a volume of fluid smaller than the volume of the original culture. The presence of much non-phage material limits the extent to which phage can be thus concentrated, for suspensions become inconveniently thick when more than a ten-fold concentration is attempted. When resuspended sediments were incubated at 25° the phage titre decreased at a rate inversely proportional to the volume of suspending fluid. For example, phage sedimented after addition of clupein sulphate to 0.05 % and suspended in a volume of nutrient medium equal to



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PLATE 1

(Facing p. 459)

one-tenth that of the original culture, showed seven times more phage activity than the original culture, but the activity fell to one-sixth after 24 hr. of incubation at 25°, while that of the original culture remained unchanged. When the sediment was suspended in a volume of the nutrient medium equal to that of the original culture, phage activity of the suspension was about 70 % of that of the original culture, and was only halved after 24 hr. of incubation at 25°. Presumably the inactivation was caused by clupein in the sediment and was correlated with its concentration. The decrease in phage activity of the suspensions was prevented by the addition of trypsin and incubation for a few hours at 37° at *c.* pH 7; but as trypsin, as well as clupein, affects phage/host interaction, the method has only slight value for fractionating and concentrating the phage.

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EXPLANATION OF PLATE

Fig. 1. The chromatograms of (from left to right): (1) intact clupein sulphate; (2) clupein sulphate hydrolysed by trypsin; (3) clupein sulphate hydrolysed by chymotrypsin, (4) clupein sulphate hydrolysed by trypsin and chymotrypsin; (5) arginine.

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Observations on the Anomalous Proteins Occurring in Extracts from Plants Infected with Strains of Tobacco Mosaic Virus

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SUMMARY: When extracts from plants infected with various strains of tobacco mosaic virus were ultracentrifuged, the non-infective supernatant fluids still contained 0.5-5 % of the protein serologically related to the viruses. The small, mostly spherical, particles aggregated to form short rods as the antigen was progressively purified by precipitation with acid or salts. It formed long rods when heated in pH 5.5 buffer or when incubated with trypsin. As the particles increased in length, their serological behaviour in precipitation tests changed from 'somatic' to 'flagellar' type.

Purified preparations of the unsedimented antigen from plants infected with either of two virus strains contained 0.1-0.2 % phosphorus, seemingly in the form of a ribose nucleic acid. No evidence was obtained that the preparations were mixtures containing some particles with the 0.5 % phosphorus characteristic of infective virus and some particles of protein free from nucleic acid.

One virus strain produced a higher ratio than the others of unsedimented to sedimented antigen. The amount of unsedimented antigen was correlated with the total content of anomalous protein when the protein was increasing rapidly, but later it fluctuated unpredictably. No conditions were found that consistently favoured its accumulation, but when plants systemically infected with the type strain were kept at 36°, the total amount of antigen decreased, while the amount unsedimented sometimes increased.

The proportion of the total antigen now obtained as poorly infective nucleoprotein is much less than 10 years ago, when a third of it sedimented in the ultracentrifuge but failed to compact into a pellet. Now the uncompacted sediment, with all the host plants and virus strains used, contains only a trivial part of the total antigen.

The virus released into sap when leaves are minced is, weight for weight, more infective than the virus that remains in the leaf residues until it is released by fine grinding.

The early work on the anomalous protein contained in virus-infected plants was done primarily to identify material that could be related to the viruses themselves, and its main aim was to make preparations with the maximum infectivity and the minimum of different components. Little attention was given to non-infective material, and the emphasis laid on the homogeneity of purified preparations served only to strengthen the general assumption that viruses multiply by the replication of an infecting particle to give a result that is uniform except for occasional variants regarded as equivalent to mutations. When we (Bawden & Pirie, 1945*a, b*) found that plants infected with the Rothamsted tobacco necrosis virus or with tobacco mosaic virus (TMV) contained more than one type of anomalous protein, we questioned the validity of this assumption, and have since argued (Bawden & Pirie, 1950*a, b*, 1952, 1953) that infection is more usefully regarded as a change in the protein metabolism

of the host cells that leads to a variety of related though not identical particles. This idea at first received little support but is now becoming more widely accepted, and other workers (Takahashi & Ishii, 1952, 1953; Commoner, Newmark & Rodenberg, 1952; Jeener & Lemoine, 1953) have found that plants infected with TMV contain more than one type of specific particle. These different particles have different sizes and so can be separated by differential centrifugation.

The small TMV particles we (Bawden & Pirie, 1945*b*) found were antigenically similar to the larger ones, but had little infectivity and precipitated with antiserum only over a narrow range of antigen/antibody ratios. Various treatments aggregated them linearly; their morphology, physico-chemical properties and serological behaviour then resembled those of the largest particles present in infective extracts, but aggregation did not enhance their infectivity.

The small particles described by other workers have many properties in common with those we separated, but they differ in one major character. Whereas our particles contained similar amounts of phosphorus to those in infective virus preparations and seemed to be nucleoprotein, all the other workers describe their particles as containing no nucleic acid. This difference could be explained by the fact that, whereas we studied material that sedimented but did not compact into a pellet when sap was ultracentrifuged, they have studied material that does not sediment.

The report of specific proteins free from nucleic acid brought infections with TMV into line with those with turnip yellow mosaic virus, which also lead to a mixture of proteins only some of which contain nucleic acid (Markham & Smith, 1949). The attention now being given to non-infective particles that are protein only is in its turn tending to oversimplify the problems concerned in infection, for the assumption is now being increasingly made that infectivity depends solely on the presence of nucleic acid. Certainly all the evidence points to its presence being essential, but specific nucleoproteins that are not infective also occur, not only in plants infected with TMV, but also in those infected with the Rothamsted tobacco necrosis virus (Bawden & Pirie, 1945*a*, 1950*a*).

The significance and origin of these various specific but not infective proteins is obscure. Equally they could be stages in the synthesis of virus particles, break-down products of virus particles, or concomitant products of protein synthesis that were never destined to become infective. While studying extracts from plants infected with the tobacco necrosis virus, we (Bawden & Pirie, 1950*a*) observed that purified preparations made from sap allowed to age at room temperature, or that were treated in some other ways, were more infective than preparations made rapidly from fresh sap kept cold. We interpreted this as evidence that the virus was gaining infectivity *in vitro*; we now know that this interpretation was wrong, for we have found that pellets sedimented from fresh sap contain systems that inactivate this virus (Pirie, 1952, 1953). These systems are unstable and soon destroyed in fresh sap, but they sediment with the virus and remain active when ultracentrifuge pellets from fresh sap are suspended in water.

With these effects in mind, we examined again sap from plants infected with TMV, to see whether components of leaf sap were acting *in vitro* to affect the infectivity of this virus and the amount of non-infective protein serologically related to it. We found no effects similar to those observed with the tobacco necrosis virus, and the infectivity of extracts containing TMV varied no more with the various treatments than could be accounted for by changes in the extent to which the particles became aggregated. In the course of these experiments we found we could no longer duplicate the results we got 10 years ago. Slowly sedimenting protein then accounted for a third of the total anomalous protein extracted from systemically infected leaves. Now it accounts for only about 1 % of it. Not only can we not repeat our own early results, but we also fail to repeat the results of other workers who describe their antigens as free from phosphorus. We have never isolated a protein that is serologically related to TMV and that is free from phosphorus.

For these reasons, we have again investigated the effects on the proportion and constitution of the small particles, of varying the duration of infection, the strain of virus used as inoculum, the age and species of the host plant, and the conditions under which the infected plants were kept. These experiments have not explained why we now get so much less slowly sedimenting material than we did 10 years ago, and although they have provided abundant evidence that its quantity can vary considerably, they have failed to provide adequate explanations for the variations.

METHODS

Viruses and host plants

Unless otherwise stated, the plants infected were of the Judy Pride variety of White Burley tobacco (*Nicotiana tabacum*), a variety that resists black root rot. We also used White Burley in our earlier work, but a variety susceptible to black root rot. We have been unable to check whether this change explains some of the differences, because we can no longer get seed of the older variety. We have also used recently another variety of White Burley, two varieties of Turkish tobacco, and tomato (*Lycopersicon esculentum*), but with all these the results resembled those with Judy Pride.

Most of our work has been with two strains of TMV, which we shall call the type strain and the *Datura* strain. Neither is a single strain of the virus, for if single local lesions in *Nicotiana glutinosa*, or small pieces of tobacco leaf which show different types of lesions, are used as inocula they produce a range of symptoms in tobacco plants. When continued as bulk cultures both give reasonably constant symptoms in tobacco, both deforming the leaves and producing mottles of various shades of green, with occasional yellow or necrotic spots. The type strain is derived from that used in 1945 and has been maintained continuously in glasshouse plants since then. It still causes the same gross symptom-picture and, although it may have altered during the years, we have no evidence that the unwitting selection of a new variant is responsible for the current small yields of non-infective material. We have on occasion

used as inoculum purified preparations of this strain made at various intervals since 1943, but the plants infected with these behaved like those infected at the same time from the stock-culture plants maintained in the glasshouse. The *Datura* strain is closely related to the type strain serologically and resembles it in most of its chemical and physical properties. It differs by giving smaller lesions in *N. glutinosa* and by giving a systemic mosaic disease in *Datura stramonium*, a plant that is killed by the type strain. Other strains we have used are those called U1 and U2 by Ginoza, Atkinson & Wildman (1954), and tomato aucuba mosaic virus. U1 is closely related serologically to the type strain and behaves like it in all the properties we have studied. U2 has only few antigens in common with the type strain and gives only local lesions in Judy Pride tobacco; it is the only strain of TMV we have encountered that infects tobacco but does not infect Kondine Red tomato plants; we have studied systemic infections in Turkish tobacco. Ginoza *et al.* (1954) reported various other differences between U2 and U1, of which perhaps the most relevant to our work is that U1 combines readily with some coloured components of tobacco sap, whereas U2 does not. Tomato aucuba mosaic virus also usually gives only necrotic local lesions in tobacco, and this we have studied in systemically infected tomato plants, which show a bright yellow mottle.

Extracts of plants systemically infected with all these strains contained slowly sedimenting components that were, weight for weight, much less infective than those that sedimented readily, but none contained them in the quantity that was usual in our earlier work. The amount of unsedimented antigen varied when different virus strains were compared on the same batch of plants at the same time, but it generally paralleled the amount of sedimented antigen, so that the ratio between the two was approximately constant. Only the *Datura* strain gave a consistently higher ratio than the type strain; although the total anomalous protein in extracts of plants infected with it was usually only half that with the type strain, two to four times as much antigen usually remained in the supernatant fluid after ultracentrifuging.

With influenza virus multiplying in the chick embryo, the ratio of non-infective (often called 'incomplete') to infective particles depends on the inoculum and is increased by increasing the ratio of the two in inocula (von Magnus, 1953). With this in mind, we have used inocula of the type strain made from slowly sedimenting particles, but the resulting infections did not differ from those caused by highly infective inocula, and extracts from the infected plants contained no greater proportion of slowly sedimenting components.

Plants were grown in compost and received supplements of inorganic nitrogen and phosphorus as needed. The total yields of anomalous protein from systemically infected plants were increased when the supplement produced a response in plant growth, but nutrition did not consistently affect the ratio of components that sediment differently.

The mean temperature in the glasshouses was about 22°. This was higher than in our earlier work, when the houses were heated only at night because fuel was scarce, but the results of experiments in which plants were kept at

different temperatures do not suggest that a difference of a few degrees in mean temperature would alone account for the change in ratio of slowly to rapidly sedimenting components. The plants were kept under natural light, but blinds were drawn according to usual glasshouse practice so that they were rarely exposed for long to full sunlight. When experiments required a constant temperature, the plants were kept in glass-walled thermostatically controlled chambers.

For routine work, plants with three or four well-developed leaves were inoculated on two lower leaves and systemically infected leaves were harvested from 2 to 6 weeks later, when they were showing pronounced symptoms. When extracts from inoculated leaves were studied, the leaves were rubbed uniformly over their whole upper surfaces with inoculum and then rinsed thoroughly with water.

Infectivity tests were made by the local-lesion method, with *Nicotiana glutinosa* as a test plant: preparations were usually tested at two dilutions, at least six half-leaves were inoculated with each dilution, and the different inocula were distributed over the test plants so that each occurred equally often on left- and right-hand half-leaves and at each leaf position.

Serological tests were made by adding 1 ml. of homologous antiserum at a constant dilution to each of a series of tubes containing 1 ml. antigen solution at various dilutions. The tubes were placed in a water-bath at 50°, with the fluid columns half immersed to ensure continuous mixing by convection. The highest dilution of antigen at which a precipitate separated after incubating for 3 hr. was taken as the precipitating end-point. When the end-point was to be used as a measure of the relative antigen content of different preparations, the preparations were all extensively aggregated before the titrations were made. The effects of aggregation on serological behaviour are described later.

Preparation of leaf extracts

To decrease the amount of fibre and avoid diluting the extracts with fluid of a relatively low virus content, the mid-ribs were cut away from the leaf blades when the infected leaves were picked. To get a uniform starting material in experiments made to compare different methods of making extracts, the leaf blades were heaped together, and the heap was cut into strips $\frac{1}{2}$ in. wide; aliquots were taken for the different treatments after the strips had been thoroughly mixed by shaking in a large vessel.

The leaf blades were usually minced in a domestic meat mincer, the sap expressed and the residue minced and pressed again. The sap was pressed out by hand through finely woven cloth into a cylinder cooled by ice. Sometimes the leaves were ground with a pestle in a mortar, or by dispersing them in a suitable fluid in a high speed mixer (MSE Top-drive macerator), or by forcing them through a slot about 0.001 in. wide (Pirie, 1956a). After grinding in the meat mincer, the residues were in a convenient state for further subdivision by the macerator or by forcing through the slot.

In attempts to prevent changes in the state of virus particles during extrac-

tion from leaf cells, we added a range of substances, such as neutralized ascorbic acid, formaldehyde, sodium azide, magnesium hydroxide, and maleate or citrate buffers in amounts enough to raise the pH value from the usual 5.5–6.1 to 6.3–6.6. None of these additions consistently affected either the ratio of sedimented to unsedimented antigen or the infectivity of the virus after isolation.

In 1945 we found that the aggregation of small particles in sap could be partly avoided by freezing the intact leaves and then, after thawing, washing away the diffusible components before grinding the leaves. We have repeated this treatment and also treated the intact leaves in other ways, such as exposure to chloroform vapour at 0° or 18°, and infiltration *in vacuo* with water, ascorbic acid or buffer solutions. None of these treatments consistently affected the distribution of antigen when the extracts were ultracentrifuged, and, when freezing the leaves had any effect, in striking contrast to our experience in 1945, it decreased the amount of unsedimented antigen.

As a routine treatment, the fresh extracts were clarified by centrifuging for 16 min. at 8000*g* and 0° and the supernatant fluids were then immediately ultracentrifuged. Experiments in which the time between making the extracts and ultracentrifuging were varied, showed that long delays invariably decreased the amount of unsedimentable antigen, whereas short delays gave variable results. Thus, when immediate ultracentrifugation produced a supernatant fluid with a precipitation end-point of 1/64, the end-point was usually 1/32 when centrifugation was delayed by a day spent at 0° and only 1/8 with a delay of several days. The precipitation end-point diminished sooner at 18° or 30°, but exposures of a few hours to these temperatures, whether or not air was excluded, did not decrease the amount of unsedimented antigen. On the contrary, in several experiments more antigen was found in the supernatant fluids when extracts were kept for 3–5 hr. at 0° before ultracentrifuging than when they were centrifuged fresh. This phenomenon is elusive, and the increase may be apparent rather than real; it may reflect the failure of our routine serological procedure, which involves aggregating the antigen by heating, to detect all the antigen in some supernatant fluids from fresh extracts, rather than an increase in the antigen in the supernatant fluid of the stored sample.

Ultracentrifugation of extracts

Our standard centrifugation was: 30 min. acceleration to reach 40,000 r.p.m. (80,000*g*), 30 min. at this speed, deceleration for 30 min. down to about 25,000 r.p.m. and then more rapid deceleration to stop in 15–20 min. The rotor was cooled to 0° before a run and was usually 4° or less after it. Centrifugation at lower speeds or for a shorter time left considerable amounts of infective material in the upper 6 ml. of the 7.7 ml. contained in the tubes.

The tubes are inclined at 10° to the axis of our centrifuge (Masket, 1941), so that sedimentation is across rather than down the tubes. Consequently, in one sense, a particle is sedimented when it has moved about 1 cm., but its final position depends on the readiness with which it slides down the wall of the

tube. Provided the initial fluid is optically clear and contains nothing that sediments readily and sticks to the walls, we have no reason to think that TMV ever fails to slide down completely.

The pear-shaped pellet of compacted antigen is overlain by an uncompacted layer that is obviously denser and darker than the bulk of the supernatant fluid. In our previous work this layer contained much of the antigen, but now its antigen content does not differ greatly from that of the upper supernatant fluid. Nevertheless, we have kept the uncompacted layer separate from the pellet and the rest of the fluid. Immediately the ultracentrifuging was ended, the top 6 ml. of fluid was siphoned off carefully, and the remaining fluid poured into a different container. The pellet was allowed to drain for a few minutes, the open end of the tube rinsed with water, and the pellet suspended in water. Further subdivision of the supernatant fluid seemed unnecessary, for, as would be expected from the lateral movement of particles during ultracentrifuging, we have never found any differences between the top 3 ml. and the next 3 ml. when these portions were siphoned off separately.

RESULTS

The measurement of antigen content by precipitation tests

Commoner & Rodenberg (1955) commented on the fact, for which they said they could offer no explanation, that preparations of the fraction they called B8 yielded smaller precipitates with its homologous antiserum than infective preparations of TMV yielded with this serum. The explanation probably lies in the different average particle size of the antigens in the two types of preparation, for although they described their B8 as a polymerized protein, their method of aggregation (exposure to pH 5) would probably still leave many particles smaller than those in normal preparations of TMV purified by the usual method. Similarly, the slight differences they described between the behaviour of infective preparations could be explained by the fact that the preparations were in different states of aggregation at the time of testing. We found (Bawden & Pirie, 1945*b*) that precipitation of this antigen by antibodies depended on the size and shape of the particles.

The TMV antigen preparation ranges from approximately spherical particles about 15 m μ . in diameter to rods more than 1 μ . long and, with preparations at extremes of this range, precipitation with one antiserum will give the contrasting features usually considered typical of 'somatic' and 'flagellar' antigens. Preparations containing only small particles give dense white floccules that separate slowly and with dilute antiserum separate only over a small range of antigen/antibody ratios around a sharply defined optimum (Table 1). Preparations containing long rods give fluffy open precipitates that separate quickly over a wide range of antigen/antibody ratios, and there is no sharp optimum unless antiserum is used very dilute. Both kinds of preparation precipitate more rapidly below than above pH 7 (Table 1).

When antigen preparations contain mixtures of particles of different sizes, or of those intermediate between the extremes, the precipitation behaviour is also

intermediate. Most of the particles in ultracentrifuge pellets that are resuspended in water at pH 6-7 have lengths distributed around 300 m μ . and behave in this intermediate way. In our conditions of testing, precipitation is usually apparent within 5 min., but in these conditions, with antiserum diluted less than 1/100, the optimum for first precipitation is poorly defined.

Table 1. *The effect of antiserum concentration and pH values on the precipitation of unaggregated and aggregated antigen by antibodies*

Antiserum dilution	pH value	Time (min.)	Dilution of unaggregated antigen						
			1/2	1/4	1/8	1/16	1/32	1/64	
1/25	6	5	+	—	—	—	—	—	
		15	++++	+++	++	+	—	—	
		45	++++	+++	+++	++	++	+	
1/100	6	15	—	—	+	—	—	—	
		45	++	+++	+++	+++	++	+	
1/400	6	45	—	—	—	—	+	—	
Dilution of aggregated antigen									
			1/2	1/4	1/8	1/16	1/32	1/64	1/128
1/400	5	5	++++	+++	++	+	—	—	—
		15	++++	+++	+++	++	++	+	—
		45	++++	+++	+++	+++	+++	++	+
1/400	6	5	+++	++	+	—	—	—	—
		15	++++	+++	++	+	—	—	—
		45	++++	++++	++++	+++	++	+	+
1/400	7	15	+	+	—	—	—	—	—
		45	+++	+++	+++	++	+	—	—

+ signs indicate the amount of precipitate. The precipitates of the unaggregated antigen were white and opaque, and those of the aggregated were grey and translucent. The time is the interval between putting the antigen-antibody mixtures in the water-bath and taking the reading.

Preparations of TMV antigen that differ greatly in their average particle size behave so differently in precipitation tests that, from a knowledge only of this behaviour, it would be reasonable to assume that they also differ widely in their content of specific antigens. This assumption is made unlikely by the fact that a preparation, which is precipitating as a typical 'somatic' antigen, will behave in a typical 'flagellar' manner with the same antiserum after the particles have been aggregated. However, as aggregation might alter the antigenicity by obscuring, freeing or destroying some antigens, we have injected rabbits separately with preparations of small particles that did not sediment at 40,000 r.p.m. and with preparations made by aggregating infective preparations. No qualitative differences were found between antisera prepared against the two types of antigen and each antiserum could be fully adsorbed by the heterologous antigen.

There is, then, nothing to suggest that the small particles differ in their antigenicity from large ones, or that increasing the length of the particles alters their antigen content. The differences in precipitation behaviour seem attributable

solely to the physical state of the antigen, which will obviously influence the measurements customarily used in serological assays. We have stressed (Bawden & Pirie, 1946) that measurements either of optimal precipitation point or of precipitation end-point can be used to assess the antigen content of TMV preparations only when these preparations contain particles in the same state of aggregation. The qualitative behaviour during precipitation tests gives some information about the state of aggregation, but the only way in which reasonable uniformity can be assured is to aggregate the preparations as fully as possible before the test is made. This, too, has the advantage that precipitation will occur more rapidly and the tests can safely be done with much more dilute antiserum.

Methods of causing aggregation

There are various ways in which preparations can be aggregated; the most reliable is to incubate them with trypsin. When many samples are to be tested, however, this is laborious, because of the need to adjust the pH value, for whereas aggregation by trypsin requires a pH value above 7, the precipitation test is best done about pH 6. With most kinds of preparation, heating at 60° and pH 5.5 is as useful as is incubation with trypsin. When using heat to aggregate the small particles in the supernatant fluids from ultracentrifuged saps, the conditions need controlling carefully, because prolonging the time at 60°, or increasing the temperature, can lead to the loss of much antigen. Our standard procedure has been to heat for 3 min. at 60° in 0.1 M-phosphate buffer (pH 5.5).

This heating aggregates the antigen adequately in unfractionated sap, in preparations made by resuspending the ultracentrifuge pellets, and in the supernatant fluids from the sap of green leaves; the titres from such preparations after heating are the same as those obtained when the preparations are incubated with trypsin. With other kinds of preparation, however, the standard heating can cause loss. Samples of supernatant fluid from ultracentrifuged sap of yellow leaves when heated fresh often give lower titres than do samples incubated with trypsin; their titres increase to those of trypsin-incubated samples when the fluid is stored for a day or more before being heated. A similar phenomenon occurs more often with the antigen present in the 1.5 ml. of fluid that overlies the pellets, and in this fraction it may happen with sap from green or yellow leaves. Some samples that gave precipitation end-points of 1/64 after incubation with trypsin, or when stored for a day before being heated, failed to precipitate with antiserum when heated fresh, but the difference is more commonly a factor of four between the samples treated differently.

We have not studied this phenomenon in detail, because, once recognized, the possible sources of error it introduces are easily avoided. All the fluids in which it occurs contain much material that coagulates at 60° and partly precipitates on ageing. This precipitation is particularly noticeable in the 1.5 ml. of fluid that overlies the pellet. We assume that the heat coagulum carries with it more antigen than does the precipitate that forms slowly in the cold, but we

have been unable to recover the antigen by incubating the heat-denatured coagulum with trypsin and there are other possible explanations. The antigen may be more than usually sensitive to heat in these fluids and be destroyed by 3 min. at 60°; or it may combine with some material in the fresh preparations to form a complex not precipitable by the antiserum, for there are other examples of 'somatic-type' antigens which form non-precipitating complexes when heated in the presence of much other protein (Bawden & Kleczkowski, 1941; Kleczkowski, 1945).

The infectivity of preparations

As a routine all the fractions separated from ultracentrifuged extracts were assayed both for their relative infectivities and their antigen contents. In addition to the regular comparisons between the sedimented pellets and the unsedimented antigen, many comparisons were also made between the pellets sedimented from different lots of sap or from leaf extracts made in different ways. Pellets from all lots of sap were highly infective, producing lesions at 100 times the dilution needed to give a visible precipitate with antiserum. By contrast, the unsedimented antigen was always poorly infective. Some supernatant fluids with precipitation end-points of 1/64 produced no lesions, and those that produced a few lesions per leaf always gave a ratio of antigen content/infectivity that was 50 or more times greater than with the sedimented antigen. Infectivity in the supernatant fluids can be avoided provided great care is taken to ensure that the cones which shut the tops of the ultracentrifuge tubes do not trap any sap, but some contamination from this source is usual when making bulk preparations. Consequently, when the antigen is concentrated by methods described below, the preparations usually contain some infective particles that should have sedimented when the sap was ultracentrifuged.

Infectivity per unit weight of the sedimented virus varied with different batches of leaf sap, but rarely by as much as a factor of two, differences too small to attempt to correlate with other variables such as duration of infection, age of leaf or cultural treatments. Purified preparations of the *Datura* strain were always, weight for weight, less infective than those of the type strain when tested on the same *Nicotiana glutinosa* plants.

Another consistent difference found with both strains of virus, and confirming earlier results with the type strain (Bawden & Pirie, 1945*b*, 1946), is that virus released into the sap when leaves are minced is more infective than the virus that remains in the leaf fibre until released by fine grinding. More of the total virus contained in leaves is released into the sap made by mincing leaves now than 10 years ago, but when tested at 1 mg./l. the virus sedimented from the sap has again produced three to four times as many lesions as the virus sedimented from extracts of the finely ground residual fibre. The same difference is found whether the fibre is ground with a pestle and mortar, a high-speed macerator, or by pressing hydraulically through a fine slot. As such different mechanisms produce the same result and the infectivity of purified virus is not affected by adding it to fibre from uninfected plants and

subjecting the mixture to the various treatments, it seems unlikely that infectivity is decreased by changes occurring during the grinding. This possibility cannot be excluded, but it seems more probable that the virus in different sites in the leaves differs in infectivity.

Variations in the yield of unsedimented antigen

Some samples of ultracentrifuged sap have given supernatant fluids with precipitation end-points of $1/256$, after aggregating the antigen. These have been commoner with sap from plants infected with the *Datura* strain than with the type strain, but even with the *Datura* strain precipitation end-points of $1/64$ and $1/32$ are more usual. The end-points are not always correlated with the total antigen in the initial sap, for although high end-points are rare unless the sap also contains much sedimentable antigen, some samples of sap with high antigen contents have yielded supernatant fluids with little unsedimented antigen. Most often the unsedimented antigen has been less than 1% of the total antigen, though with the *Datura* strain it has sometimes been as much as 5%. Differences in the amount of unsedimented antigen can only be measured in the supernatant fluids. Many of the variations may be caused by antigen under some circumstances appearing in the supernatant fluid and in others being sedimented, but the amount sedimented is so large that this possibility cannot be tested experimentally, because differences too small to measure in the sedimented antigen would account for the whole of the unsedimented antigen.

The yields of unsedimented antigen from successive batches of leaves intended to be similar have varied considerably. We think it unlikely that differences in centrifugation account for these variations, because extending the period of centrifuging much beyond that needed to sediment all the infective particles, or recentrifuging a supernatant fluid, does not decrease the amount of antigen that remains unsedimented. It is more likely that some uncontrolled variation in the condition of the plants affects the state of the antigen or the composition of the extract.

We have studied several variables that affect the total amount of antigen in leaf extracts without being able to correlate the amount unsedimented with any one, except with duration of infection in newly infected leaves. As found by Commoner & Rodenberg (1955), infective virus and sedimentable antigen occur in inoculated leaves before any unsedimented antigen is detectable. This does not necessarily mean that the virus is produced first; if sedimentable and unsedimented antigen were being produced simultaneously in the ratio in which they occur later, the unsedimented antigen would not be detected by our methods until the precipitation end-point of sap exceeds $1/100$. Clearly the unsedimented antigen is not formed in bulk as a precursor for the large particles; if it is in any way a precursor, as suggested by van Rysselberge & Jeener (1955), it soon changes to larger particles when the virus content of infected leaves is increasing rapidly. As the total virus content of infected leaves increases, so also for a time does the content of unsedimented antigen,

but then it is apt to fluctuate in successive batches from the same plants. When we have studied plants systemically infected for different periods, however, we have usually obtained more from those infected for 3 weeks than from those infected for 6 weeks.

Table 2 shows the results of one experiment comparing the antigen content of systemically infected leaves occupying different positions on the main stem and picked 3 weeks after the plants were inoculated. Consistently, sap from

Table 2. *The content of sedimented and unsedimented antigen in systemically infected leaves of different ages*

Leaves were picked 24 days after plants were inoculated with the type strain of TMV, the sap extracted and ultracentrifuged. The supernatant fluids were titrated against TMV antiserum and their antigen content is given as the reciprocal of the maximum dilution at which precipitation occurred. The sedimented virus was purified and weighed.

Type of leaf	Unsedimented antigen (relative contents)	Sedimented antigen (g./l. sap)
Youngest, < 5 cm. broad	64	3.6
Intermediate, 5-8 cm. broad	64	2.2
Intermediate, > 8 cm. broad, green	8	1.9
Oldest, > 8 cm. broad, yellow	4	1.2

the oldest leaves contained least total antigen, but the diminution in the amount of unsedimented antigen from young to old leaves shown in this experiment was not reproduced in other experiments. The reporting of results as antigen/ml. sap can be misleading, for those in Table 2 may suggest that young leaves contained more virus than did old ones. This is not so, the oldest leaves weighed five to ten times more than the youngest and also gave more sap for equal fresh weights, so that their total antigen content was much greater.

The fluctuations in content of unsedimented antigen suggest that this antigen is unstable and that conditions in the host cells before the leaves are picked may affect its amount. Exposure to temperatures around 36° can free infected plants from some viruses, and Kassanis (1954) showed that leaves systemically infected with TMV contain less virus when plants are kept at 36° than when kept at 20°. As our results seemed to vary more in the summer, when the temperature sometimes reached 30° or more in the glasshouse, than in the winter, we have kept systemically infected plants at different temperatures before they were harvested. Plants kept at 36° for a week or more before the leaves were picked have always given less sedimentable antigen than those kept at mean temperatures of 24° or lower, but they have sometimes given more unsedimented antigen. To quote one experiment in which plants already systemically infected with the type strain were kept for 10 days in three different conditions, the yield of sedimented antigen was 4.1 g./l. sap from plants at 17°, 3.8 at 24° and 1.7 at 36°, whereas the relative yields of unsedimented antigen (expressed as reciprocals of their precipitation end-

points) were 32, 64 and 128. In other experiments, however, though the amount of sedimented antigen decreased, the amount unsedimented was not increased by high temperatures.

Purification of the unsedimented antigen

The starting material for purifying the antigen was, perforce, the supernatant fluids from leaf extracts centrifuged at 40,000 r.p.m., and producing the antigen in the amounts needed for critical examination entailed re-loading the centrifuge many times. To avoid this tedious ultracentrifuging we tried to concentrate the unsedimented antigen by such treatments as pressing water from unminced leaves that had been frozen and thawed, or concentrating the total antigen in extracts by partial freezing, by dialysis under pressure, or by precipitating it with acid or salt and resuspending in a smaller volume. None of these treatments was successful, and when such preparations were ultracentrifuged, although their supernatant fluids usually contained some unsedimented antigen, this was never more concentrated than in the original extract. These treatments presumably either aggregate the small particles so that they sediment in the ultracentrifuge or attach them to some leaf or sap component.

After centrifuging at 40,000 r.p.m., the upper 6–6.5 ml. of fluid in each tube were collected and kept at 0°. By the time three or four loads had been centrifuged, the first fluids contained some precipitate, but all were mixed, adjusted to pH 4.6 and centrifuged at 8000 r.p.m. immediately. On adjusting the fluid to pH 3.3 a shimmer developed but when centrifuged at this stage the precipitate packed badly; after standing at 0° for a few hours the fluid was stirred briefly and left undisturbed overnight for the precipitate to settle. Most of the fluid was poured off. The white precipitate contained 80–90 % of the original antigen and was collected by centrifuging at 8000 r.p.m. The remainder of the antigen was mainly in the first precipitate that separated at pH 4.6, though some was in the fluid poured off at pH 3.3. The antigen in the precipitate can be obtained by incubation with trypsin at pH 7, separating the material that still precipitates at pH 4.6, and then precipitating at pH 3.3. The antigen in the fluid poured off at pH 3.3 can be precipitated by adding $(\text{NH}_4)_2\text{SO}_4$ at the rate of 400 g./l.

The main precipitate at pH 3.3 was suspended in a volume of water equal to 1/100 the original supernatant fluid, neutralized and clarified by low-speed centrifugation. The precipitation at pH 3.3 was repeated to remove contaminants carried over mechanically at the first precipitation. The antigen solution was then ultracentrifuged; although none of the antigen originally sedimented at 40,000 r.p.m., some then sedimented, for the small particles progressively aggregated during precipitation with acid. When ultracentrifuged between pH 6.5 and 7.5, about 10 % remained in the upper 6 ml. of supernatant fluid, and the bulk was equally divided between a compacted pellet and an uncompacted sediment overlying it. As observed by Commoner & Yamada (1955), the antigen sediments more readily from acid solutions, and at pH 5.5 almost all of it compacts into the pellet. The addition of salts, e.g. 0.1M-ammonium

acetate, also makes sedimentation from neutral solutions almost complete. Antigen that has compacted in these conditions is not permanently altered and when again ultracentrifuged from water at pH 6.5–7.5, part again remains uncompacted.

Any infective particles that may have been present as contaminants in the original supernatant fluid are concentrated into the pellet, leaving an almost non-infective, uncompacted layer. Typically, a solution of the uncompacted layer containing 50 mg. antigen/l. may give an average of fewer than 1 lesion/half-leaf, when the pellet at the same concentration gives 10 or more lesions and the virus that sedimented from sap 20 or more lesions at only 1 mg./l. For most purposes this fractionation is unnecessary, because except for slight differences in serological behaviour, which suggest that the pellet contains particles of a slightly larger average size, we have found no other differences between the compacted and the uncompacted sediment. However, all results obtained with unfractionated preparations were confirmed on preparations that were either uninfected from the beginning or were made so by fractional centrifugation.

We have used this method with equal success to make preparations of the small antigens contained in extracts from plants infected with either the type strain or the *Datura* strain of TMV. There is no call to distinguish between the two, for although each contains some specific antigenic groups, their general physical and chemical properties are closely similar. We have made no preparations of the small antigens from the other strains.

The purified preparations of small antigens precipitate more rapidly with TMV antisera than do the small particles in the supernatant fluids from ultracentrifuged samples of fresh sap, but less rapidly than normal virus preparations. Few or no rods are seen when the supernatant fluids are examined in the electron microscope, whereas purified preparations contain few spheres and most of the material occurs as rods approximately 15 m μ . wide and of various lengths between 30 and 200 m μ . The distribution of particle lengths was determined in some preparations; the mean was about 75 m μ . and the most common length 50 m μ . When such preparations were heated with pH 5.5 phosphate buffer, or were incubated with trypsin, the particles aggregated linearly and their physical and serological behaviour was much altered. Solutions containing 1 g./l. then showed anisotropy of flow strongly and electron microscopy showed many long particles, with an average length of more than 300 m μ . Morphologically, and serologically, the preparation was now indistinguishable from one of aggregated infective virus. It precipitated rapidly with virus antiserum, precipitated over a wide range of antigen-antibody ratios and gave a precipitation end-point of about 3 mg./l., compared with 12 mg./l. given by the same preparation before aggregation. Like infective TMV, the non-infective antigen resisted hydrolysis by commercial preparations of proteolytic enzymes and by air-borne bacterial contaminants.

Preparations of infective virus contain 0.5 % phosphorus. Our preparations of the non-infective antigen contained between 0.1 and 0.2 % P, and we gave considerable attention to finding how this is combined because other workers

(Takahashi & Ishii, 1952; Commoner *et al.* 1952; Jeener & Lemoine, 1953) were unanimous that their unsedimented antigen was not a nucleoprotein and contained no phosphorus. Repeated precipitation at pH 3.3 or from neutral solutions with ammonium sulphate, or sedimentation in the ultracentrifuge, did not alter the P content of preparations, and when sedimented in conditions which gave a compacted pellet and an uncompacted sediment, the P content of the two fractions did not differ by an amount exceeding the experimental error of determinations on small amounts of material containing so little P (Holden & Pirie, 1955*b*). Preparations were incubated with commercial trypsin, or with pancreatic or leaf ribonucleases in the presence of citrate, and when the aggregated antigen was recovered by ultracentrifugation its P content was unchanged. These treatments did not separate the phosphorus in a form unprecipitable by trichloroacetic acid; they destroyed the normal leaf nucleoprotein that often contaminates preparations of TMV (Pirie, 1956*b*), and so it is unlikely that the phosphorus is present as this type of contaminant.

The phosphorus appears to be present as nucleic acid because it is split off by heating at 100°, or by extracting at 20° with N-HClO_4 , in the same manner as nucleic acid is split from TMV (Pirie, 1956*b*). These extracts have a characteristic UV absorption maximum at 260 $\text{m}\mu$. The absorption maximum of the antigen itself is at 275 $\text{m}\mu$., which is not unexpected considering the preponderating part that absorption at 280 $\text{m}\mu$., the usual maximum for proteins, will play in a protein with such a small content of nucleic acid. These observations exclude the possibility that the phosphorus is present as a contamination by phosphate, metaphosphate or most of the phosphoric esters. As they do not exclude deoxyribonucleic acid, which is present in some TMV preparations (Hoff-Jørgensen, 1952; Holden & Pirie, 1955*a*), HClO_4 - extracts of the antigen were neutralized with KOH, filtered, evaporated to dryness, and tested for deoxyribonucleic acid by the Dische (1955) method. Using conditions under which 20 μg . deoxyribonucleic acid can be measured satisfactorily, none was found in samples with a P content and UV absorption corresponding to 300 μg . nucleic acid. In comparisons between this type of extract and extracts with the same P content made from normal TMV, the same colours were given by the Bial reaction for pentoses (Dische, 1955). We have had too little material to be able to isolate nucleic acid and recognize its characteristic physical properties, but the P in extracts made by boiling the antigen was largely precipitated by acid and lost this property after incubation with leaf ribonuclease.

All this evidence suggests that the phosphorus in these antigen preparations is present as ribonucleic acid and that the link between this and protein is similar to the one in TMV, for each is stable in a range of conditions that disrupt many other nucleoproteins. An obvious interpretation for a content of 0.2 % P is that our preparations of unsedimented antigen were mixtures, consisting of material that is two-fifths nucleoprotein with the usual content of 0.5 % P and three-fifths of protein free from nucleic acid; but we have been unable to get any evidence to support this. Not only have we failed to frac-

tionate the preparations by many different methods of precipitation, but when examined electrophoretically they gave a single peak, whereas mixtures of the antigen and infective preparations of TMV gave two peaks.

DISCUSSION

The main points that call for discussion are the differences between our present and past results and between our results and those of other workers. There is no certain explanation, but it seems likely that the range of anomalous proteins in plants infected with strains of TMV can be greater than previously recognized and that the extent to which the different types accumulate differs greatly in different conditions. The main component is always a nucleoprotein with large, readily sedimentable particles and, except for some differences in particle length, this seems reasonably uniform. However, there are many methods (Bawden, 1950) whereby virus preparations can be rendered non-infective without any obvious change in their physico-chemical properties or serological behaviour, and preparations partly inactivated by such treatments have not yet been separated into infective and non-infective portions. Until some method of fractionating such a mixture is developed, the possibility cannot be excluded that ordinary preparations of TMV are mixtures of infective and non-infective particles. Indeed, the fact that preparations made at different times by similar methods differ in their infectivity per unit weight, although they resemble each other in other properties, suggests that the preparations contain different proportions of infective and non-infective particles. In our earlier work we demonstrated that much of the nucleoprotein had little infectivity. This protein did not compact into a pellet when sap was ultra-centrifuged and so could be separated from the more-infective protein that did. There has been little of this uncompacted sediment in extracts we have centrifuged recently, but this does not necessarily mean that there is also only little of the non-infective nucleoprotein. It may occur in the same amounts as previously, but in a different condition so that it now compacts and the previous method of fractionation is no longer effective. The change may be in the composition of our plants or extracts, which previously allowed this material to accumulate as small particles but now does not.

Although we can not define conditions that encourage the smallest particles to accumulate, our results show that their amount varies considerably with the virus strain used, the duration of infection and with the conditions under which the plants are grown. Our results, too, agree with those of other workers in that this unsedimented protein differs chemically from the sedimentable protein, even though we find some phosphorus and they found none. Different conditions of the host plant or different virus strains may also account for this difference, because specific products of virus multiplication may accumulate preferentially in different conditions.

None of the results of detailed examination of the anomalous proteins present in extracts of plants infected with TMV fits with the idea of virus multiplication leading to a uniform, stable end-product. They are more easily

interpreted by regarding infection as a change in the protein metabolism of the host, with the conditions in the cells determining the equilibrium between the various products of metabolism. Harrison (1956) found that the Rothamsted tobacco necrosis virus can multiply at 30°, and that at this temperature it is also inactivated in the leaves. The results obtained when plants systemically infected with TMV were kept at 36° suggest that this virus, too, is inactivated at high temperatures, and that the concentration at any given time probably represents an equilibrium between synthesis and degradation. In this respect, at least, viruses probably resemble the normal leaf proteins, which also vary with different conditions of growth. In the course of synthesizing and degrading virus particles, the infected cell produces proteins of a variety of types; one type becomes detectable only when it differs in some way that allows it to be separated from the bulk product and when it is stable enough to accumulate. A change in conditions will change the relative activity of different enzyme systems in the cells, and the types of protein that are stable in one set of conditions will not necessarily be the same as those stable in another.

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The Kinetics of Urease Activity in *Corynebacterium renale*

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SUMMARY: The urease activity of *Corynebacterium renale* has been studied in washed suspensions and in cell-free extracts of the micro-organism. The urease is constitutive, and whole organisms have a urease activity of 17,000-22,000 $\mu\text{g. NH}_3/\text{mg. dry wt. organisms/hr.}$ in 0.5 M-urea in phosphate buffer at pH 7.0. In a cell-free extract the enzyme is optimally active at pH 7.5, has a Michaelis constant of 0.030 M, and a temperature velocity constant of 7800 cal.; the activity is inhibited by atmospheric oxygen and by thiourea, but not by sixteen other analogues of urea tested.

Corynebacterium renale is the causative organism of a specific bovine pyelonephritis. Observations made on natural infections in cattle and on experimental infections in mice and rabbits have established that there is a specific localization of the micro-organism in the medulla of the kidney (Lovell, 1946; Lovell & Cotchin, 1946; Feenstra, Thorp & Gray, 1949). The work presented in this paper is part of an attempt to establish a relationship between the biochemical characteristics of the micro-organism and the specificity of the infection which it produces.

Following reports that *Corynebacterium renale* produced ammonia when grown in urine, Lovell & Harvey (1950), using suspensions of acetone powders of the micro-organism, investigated the production of ammonia from amino acids and several other nitrogenous substances found in urine, and showed that the rate of production of ammonia from urea was very much greater than that from any other substrate tested. Whole organisms of *C. renale* have, in fact, a very high urease activity with a q_{NH_3} value (expressed as $\mu\text{g. of ammonia produced/mg. dry wt. organisms/hr.}$) of about 20,000, which is higher than the catalase activity of some micro-organisms. A study has been made of the kinetics of the activity of this urease as a preliminary to the investigation of the role of the enzyme in the aetiology of pyelonephritis.

METHODS

Organism and growth medium. The organism used was *Corynebacterium renale* strain 4, kindly supplied by Prof. R. Lovell of the Royal Veterinary College. It was grown in Roux bottles (150 ml. medium/bottle) in a liquid medium of the following composition (% w/v): peptone (Oxoid) 1; Lab Lemco 1; Marmite 0.1; NaCl 0.5; Tween 80 0.1.

Suspensions of organisms and cell-free extracts. Organisms for the estimation of urease activity were grown for 24 hr. at 37°, harvested by centrifugation, washed twice in 0.1 % (w/v) Na_2S adjusted to pH 7 with dilute acetic acid

(Na_2S solution), and suspended in Na_2S solution at a density of approximately 0.5 mg. dry wt./ml. To obtain a cell-free extract, a suspension of organisms of density c. 10 mg. dry wt./ml., prepared as above, was broken in the Hughes press (Hughes, 1951) and centrifuged at 10,000*g* for 30 min. to remove cell debris. The supernatant fluid was diluted with 500 vol. of Na_2S solution before use.

Estimation of urease activity. Urease activity was measured in two ways. In the first method, the procedure of Krebs & Henseleit (1932) was modified by gassing the manometers with H_2 or N_2 . In the second method (Sumner & Myrbäck, 1951), 1 ml. of m-urea in phosphate buffer (pH 7.0) was incubated at 37° with 1 ml. of a urease preparation. After a suitable period of time, usually about 20 min., when not more than 3 % of the urea had been broken down, 1 ml. N-HCl was added to stop the reaction. A sample (2 ml.) of the reaction mixture was brought to a final pH value of about 9.2 with borate buffer, and distilled in the Parnas apparatus (Parnas & Heller, 1924). The ammonia collected was estimated colorimetrically with Nessler's solution, using the Spekker absorptiometer with Ilford 601 filters. For blank values the above procedure was followed, except that 1 ml. N-HCl was added before addition of the enzyme. For each determination of urease activity, enzyme was added to ten identical tubes at 20 sec. intervals, and the average of the results obtained was corrected by the subtraction of a blank value determined in duplicate.

RESULTS

The estimation of urease activity

The urease activity of *Corynebacterium renale* proved very difficult to estimate with accuracy owing to its rapid inactivation by atmospheric oxygen. When a washed suspension or a cell-free extract was shaken in a manometer flask gassed with air, a rapid and progressive loss of activity resulted. There was no progressive loss of activity when the manometers were gassed with H_2 or N_2 , but the standard error of a set of ten identical estimations was as high as 10 % of the mean value, and this error was not decreased by introducing a small piece of yellow phosphorus into the centre well of the manometer vessels, by suspending the organisms in sodium mercaptoacetate (thioglycollate) or cysteine, or by washing the manometer vessels in versene to avoid inactivation by heavy metals. However, to minimize aerobic inactivation, cells were routinely washed and suspended in Na_2S solution.

Treatment of organisms with the detergent cetyltrimethylammonium bromide (CTAB), at a concentration of 30 $\mu\text{g.}/\text{mg.}$ dry wt. organisms, increased the urease activity threefold. Under these conditions, the standard error of ten determinations fell to 3 %. It is suggested that a considerable part of the standard error of 10 % may have been due to the lysis of different quantities of organisms in each manometer vessel. For this reason all the kinetic determinations reported in this paper were performed on cell-free extracts of *Corynebacterium renale*. The second method of estimation (above) was used because it is inconvenient to carry out a large number of simultaneous

determinations in manometers. A reliable estimate of urease activity was obtained by taking the average of ten identical estimations in each case. That this method provided a satisfactory and accurate estimate of urease activity is illustrated in Fig. 1, which shows the rate of production of ammonia from 0.5 M-urea at pH 7.0 by the cell-free extract. Despite the scatter of the results (shown by the vertical bars above and below each point), the mean values approximate well to the best straight line drawn through the origin.

The urease activity of whole organisms and of cell-free extracts

The urease activity of *Corynebacterium renale*, assayed in 0.5 M-urea at pH 7.0, had a q_{NH_3} value ($\mu\text{g. NH}_3$ produced/mg. dry wt. organisms/hr.) varying between 17,000 and 22,000. The enzyme is constitutive: it is possessed by organisms which have been grown in media containing no urea, and furthermore, the activity was not increased by growth in media containing urea, or by varying the pH value of the growth medium between 6.5 (the pH value below which the micro-organism does not grow) and 9.0. When a washed suspension of organisms was incubated with urea for a time sufficient for the reaction to reach completion, 2 molecules of ammonia were produced from 1 molecule of urea.

The enzyme was present in a soluble form in a cell-free extract, and centrifugation in the Spinco ultracentrifuge at 150,000 g for 45 min. to remove small particles resulted in no loss of activity. The extract was stable in Na_2S solution for at least 3 hr. at 4° , but was slowly inactivated after longer periods at this temperature; it was rapidly inactivated by freezing to -15° and by dialysis. In this latter respect it resembles the urease of *Bacillus pasteurii* described by Larson & Kallio (1954). An attempt was made to purify the enzyme by the procedures described by these authors, and at each fractionation material active as urease was separated from an inactive residue. However, even in the presence of Na_2S solution, each step was accompanied by a marked decrease in total urease activity, and after removing 97.3 % of the initial dry matter by the above procedures the purification was only 2.1-fold, based on the ratio urease activity/dry wt. after dialysis.

The kinetics of the cell-free urease preparation

Over the range tested the optimum pH value of the enzyme was 7.5, but there was little diminution in activity over the range of pH values 6–8 (Fig. 2).

The effect of temperature on urease activity, determined in 0.5 M-urea in phosphate buffer (pH 7.0) between 15° and 45° , is shown in Fig. 3. The values of the temperature coefficient (Q_{10}) of the enzyme at different temperatures were: 1.63 ($15-25^\circ$); 1.51 ($25-35^\circ$); 1.47 ($35-45^\circ$). The data of Fig. 3 were used to plot \log_{10} (rate of urease-catalysed reaction) against the reciprocal of the absolute temperature (Fig. 4). When the gradient of the best straight line drawn through these points is multiplied by the factor $-R \ln 10$, where R is the gas constant, the value obtained (7800 cal.) is the temperature-velocity constant of the reaction over the range $15-45^\circ$. It is possible that the points given

in Fig. 4 may lie on two non-parallel intersecting straight lines with a critical point at about 27° . Such a critical point has been described by Sizer (1939, 1940, 1941) for the ureases of soybean and of whole *Proteus vulgaris*, and by Larson & Kallio (1954) for the purified urease of *Bacillus pasteurii*. However, Fig. 4 shows that in the case of *Corynebacterium renale* the change in the gradient of the line, if any, is only small, and because of the errors involved in the estimation no attempt was made to elucidate this point.

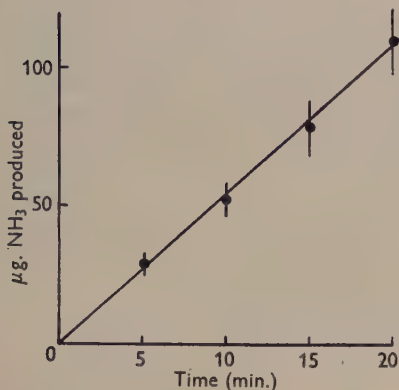


Fig. 1

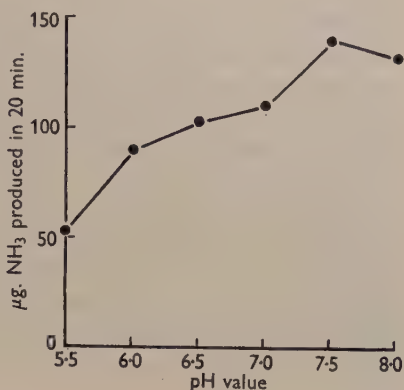


Fig. 2

Fig. 1. The time course of the production of ammonia from 0.5M-urea by a cell-free extract of *Corynebacterium renale*. The reaction was carried out at 37° in phosphate buffer (pH 7.0). The vertical bars above and below each point represent the range of 10 identical estimations.

Fig. 2. The effect of pH value on the urease activity of a cell-free extract of *Corynebacterium renale*. The data represent the rate of production of ammonia from 0.5M-urea in 0.06M-phosphate buffer at 37° .

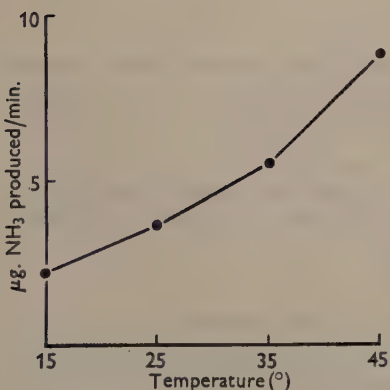


Fig. 3

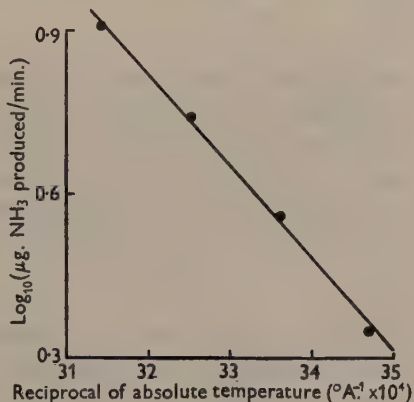


Fig. 4

Fig. 3. The effect of temperature on the urease activity of a cell-free extract of *Corynebacterium renale*. The data represent the rate of production of ammonia from 0.5M-urea in phosphate buffer (pH 7.0).

Fig. 4. Calculation of the temperature velocity constant of the urease-catalysed reaction from the data of Fig. 3.

The effect of substrate concentration on urease activity was determined at 37° in phosphate buffer (pH 7.0), and the reciprocal of the urease activity was plotted against the reciprocal of the urea concentration (Lineweaver & Burk, 1934) (Fig. 5). The intercept with the abscissa of the best straight line drawn through the points gives the Michaelis constant of the enzyme. This straight line was calculated statistically, using the results of all the individual determinations, and not only their mean values. In this way it was possible to calculate not only the mean value of the Michaelis constant of the enzyme, which is 0.030 M, but also its 95 % confidence limits of 0.026–0.035 M. This value may be compared with the values which have been obtained for the ureases of other organisms and which vary between 0.10 M for *Bacillus pasteurii* urease in 0.2 M-phosphate buffer (pH 5.7) at 25° (Larson & Kallio, 1954), and 0.003 M for crystalline jack bean urease in maleic acid buffer (pH 7.0) at 25° (Harmon & Niemann, 1949).

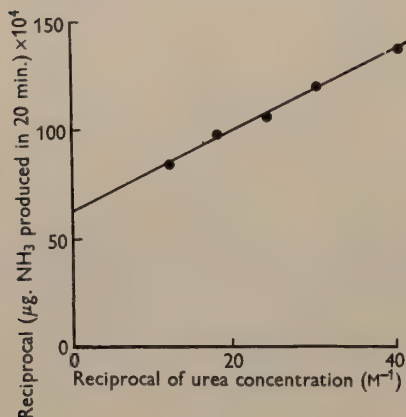


Fig. 5

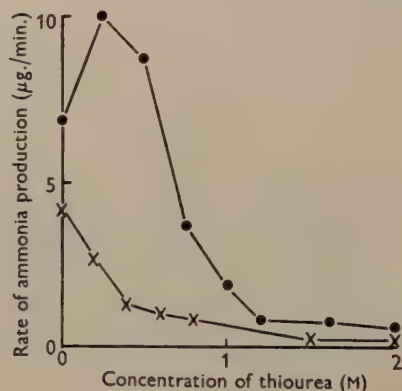


Fig. 6

Fig. 5. The effect of urea concentration on urease activity of a cell-free extract of *Corynebacterium renale*. Lineweaver & Burk (1934) plot of reciprocal of rate of ammonia production ($\mu\text{g. NH}_3/20 \text{ min.}$ from 0.5 M-urea in phosphate buffer, pH 7.0, at 37°) against reciprocal of urea concentration (M^{-1}).

Fig. 6. The effect of thiourea concentration on the urease activity of a cell-free extract of *Corynebacterium renale*. The data represent the rate of production of ammonia from 0.5 M-urea in phosphate buffer (pH 7.0) at 37°. ●—●, in the presence of 5×10^{-4} M-cysteine; ×—×, in the absence of cysteine.

The effect of urea analogues on urease activity

An investigation was made of the effect of seventeen analogues of urea upon the urease activity of *Corynebacterium renale* in order to determine whether any such analogue might be used as an *in vivo* inhibitor of urease activity. Each compound was added either in saturated solution in the case of the relatively insoluble derivatives (marked with an asterisk below), or at a concentration of 0.1 M for the others, to a cell-free solution of the urease at pH 7.0, with and without urea. The urea concentration used in these experi-

ments was 0.025M in order to enhance any competitive inhibition by the analogues. Thiourea was the only analogue tested which had an inhibitory action on urease; the following sixteen compounds were not inhibitory: *N,N'-acetylmethylurea; *N-(2-bromo-2-ethylbutyryl)urea (Carbromal); ethylurea; N,N'-(2-diethylmalonyl)urea (barbitone); methylurea; *N,N'-dimethyl - N,N' - diphenylurea; * α - naphthylurea; *phenylurea; *N,N'-diphenylurea; *N,N-diphenylurea; *o-tolylurea; *m-tolylurea; *N,N'-di-o-tolylurea; *N,N'-di-m-tolylurea; *N,N'-di-p-tolylurea; biuret. Ammonia was not produced from any of the compounds.

The effect of different concentrations of thiourea on the urease activity of a cell-free extract in 0.5M-urea was determined in the presence and absence of 5×10^{-4} M-cysteine (Fig. 6). In each case the enzyme was inhibited by the higher concentrations of thiourea, but in the presence of cysteine the urease activity was increased by 0.25 and 0.50M-thiourea. Sizer & Tytell (1941) showed that the activity of crystalline jack bean urease was affected by changes in the oxidation-reduction potential of the test system. Thiourea has a higher oxidation-reduction potential than cysteine; perhaps the activation of *Corynebacterium renale* urease by thiourea at low concentrations is due to an adjustment of the pH of the system to a value nearer to that which is optimal, while at higher concentrations this activation is outweighed by the inhibitory action of thiourea.

DISCUSSION

This study of the urease of *Corynebacterium renale* has shown that it differs from the other ureases derived from plant and bacterial sources only in minor details. It has been reported that the activity of jack bean urease is markedly dependent on the nature of the inorganic ions present in the test system (Howell & Sumner, 1934; Harmon & Niemann, 1949), and while the reasons for this difference in behaviour remain obscure, it is not possible to make a detailed comparison between the various ureases which have been isolated. The chief difference between *C. renale* urease and those ureases which have been described by other workers is that the *C. renale* urease activity/pH curve (Fig. 2) is very flat, whereas a similar curve for plant urease has a sharp peak at the optimum pH value (Howell & Sumner, 1934). It may be significant that the activity of the urease of *C. renale* is very high between pH 6.5, which is the approximate pH value of blood and also the pH value below which this micro-organism cannot grow, and pH 8.0 which is the approximate pH value of bovine urine. The *C. renale* enzyme is, in fact, very active at any pH value that this micro-organism is likely to encounter in the bovine kidney. If the *C. renale* urease has an activity of the same order, on a weight for weight basis, as that of crystalline jack bean urease, then this one enzyme would account for 0.1-1% of the total dry weight of *C. renale*. It is possible that the pathogenicity of *C. renale* is due to the local accumulation of high concentrations of ammonia in the kidney, but the kinetic data give no indication of the part played by the urease in the aetiology of pyelonephritis.

The author wishes to express his gratitude to Professor R. Lovell of the Royal Veterinary College (who suggested this problem) and Dr E. F. Gale, F.R.S., for much helpful advice and encouragement; to Mr R. G. Carpenter of the Department of Human Ecology in the University of Cambridge for assistance with the statistical calculations; and to the Medical Research Council for a Scholarship for Training in Research Methods.

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Saccharomyces pretoriensis n.sp.—from South African Soil

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SUMMARY: A new *Saccharomyces* species has been isolated from soil. It is distinguished from other species of the genus by its ability to ferment glucose, galactose, sucrose, maltose and raffinose 1/3, as well as by its small cells and the formation of protuberances resembling conjugation tubes during sporulation.

In a recent survey of the yeast flora associated with surface soil, a round-celled sporogenous strain was isolated. Characteristic of the strain was the formation, during sporulation, of protuberances resembling conjugation tubes.

METHODS AND RESULTS

The following description is based on the standard procedures described by Lodder & Kreger-van Rij (1952).

Description

Growth in malt extract. After 3 days at 25° the cells are round (2.6-6.8 μ .), single or in pairs. After 1 month at 17° a sediment is formed.

Growth on malt agar. After 3 days at 25° the cells have the same shape and size as those in malt extract. After 1 month at 17° the streak culture is soft, dull, glistening and smooth, brownish to cream-coloured. Margin smooth.

Slide cultures. No pseudomycelium is formed.

Sporulation was examined on malt agar and the common sporulation media. Isogamous or heterogamous conjugation may occur. Ascus formation without immediately preceding conjugation also occurs. Cells with protuberances are formed. Spores are round, usually with an internal oil drop. One to four spores are formed (Pl. 1, fig. 1).

<i>Fermentation.</i>	Glucose	+	Maltose	+
	Galactose	+	Lactose	—
	Sucrose	+	Raffinose	1/3
<i>Sugar assimilation.</i>	Glucose	+	Maltose	+
	Galactose	+	Lactose	—
	Sucrose	+		

Assimilation of potassium nitrate. Absent.

Ethanol as sole source of carbon. Growth.

Splitting of arbutin. Absent.

DISCUSSION

As the organism forms no pellicle, ferments glucose vigorously and cannot assimilate nitrate, it must be classified as a species of genus *Saccharomyces* (Meyen) Reess as diagnosed by Lodder & Kreger-van Rij (1952). Bio-

chemically, this strain shows great similarity to *Saccharomyces cerevisiae* in its fermentation of glucose, galactose, sucrose, maltose and raffinose for one-third. Morphologically the comparatively small round cells, the round spores with endogenous lipid globules, and especially the formation of protuberances, are again features which the strain from soil has in common with the group of *Saccharomyces* species formerly classified as *Torulospora*, viz. *Saccharomyces delbrueckii*, *S. rosei* and *S. fermentati*. The morphological resemblance to *S. fermentati* is particularly marked. Since mature cells of the strain from soil are considerably smaller than those of *S. cerevisiae*, it cannot be classified as this species. Similarly, it cannot be identified with either *S. delbrueckii*, *S. rosei* or *S. fermentati*, since none of these species ferments or even assimilates galactose. The organism must therefore be regarded as representing a new species. The name *S. pretoriensis* is proposed for Pretoria where the yeast was isolated.

A subculture of *S. pretoriensis* has been deposited in the Yeast Collection of the Centraal Bureau voor Schimmelcultures in Delft.

LATIN DIAGNOSIS

Saccharomyces pretoriensis sp.nov.

In musto maltato cellulae rotundae (2.6–6.3 μ .), singulae aut binae. Sedimentum formantur.

In agaro maltato cellulae formae et dimensiones cellularum eadem sunt que in musto maltato.

Cultura (post unum mensem, 17°) mollis, parum nitens, glabra, albiflava. Margine glabro.

Pseudomycelium nullum.

Copulatio cellularum aequarum inaequarumque asci plerumque conformationi praecedit. Etiam asci conformationi sans copulatione praecedit. Cellulae cum tuberis similibus tubulis conjugationibus.

Ascosporae rotundae; globulos olei continent, 1–4 in asco.

Fermentatio glucosi, galactosi (lente) sacchari, maltosi et raffinosi pro tertia parte. In medio minerali cum glucoso, galactoso, saccharo et maltoso crescit. Nitras kalicus non assimilatur. In media minerali cum alcohole aethylico crescit. Arbutinum non finditur.

The authors' thanks are due to Professor A. Pijper for his kind assistance with the photography. This paper is published with the permission of the South African Council for Scientific and Industrial Research.

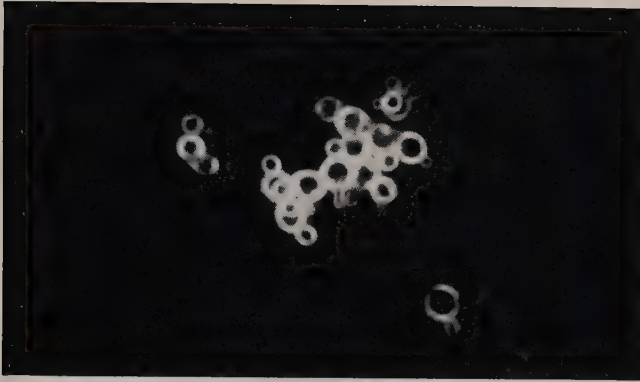
REFERENCE

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EXPLANATION OF PLATE

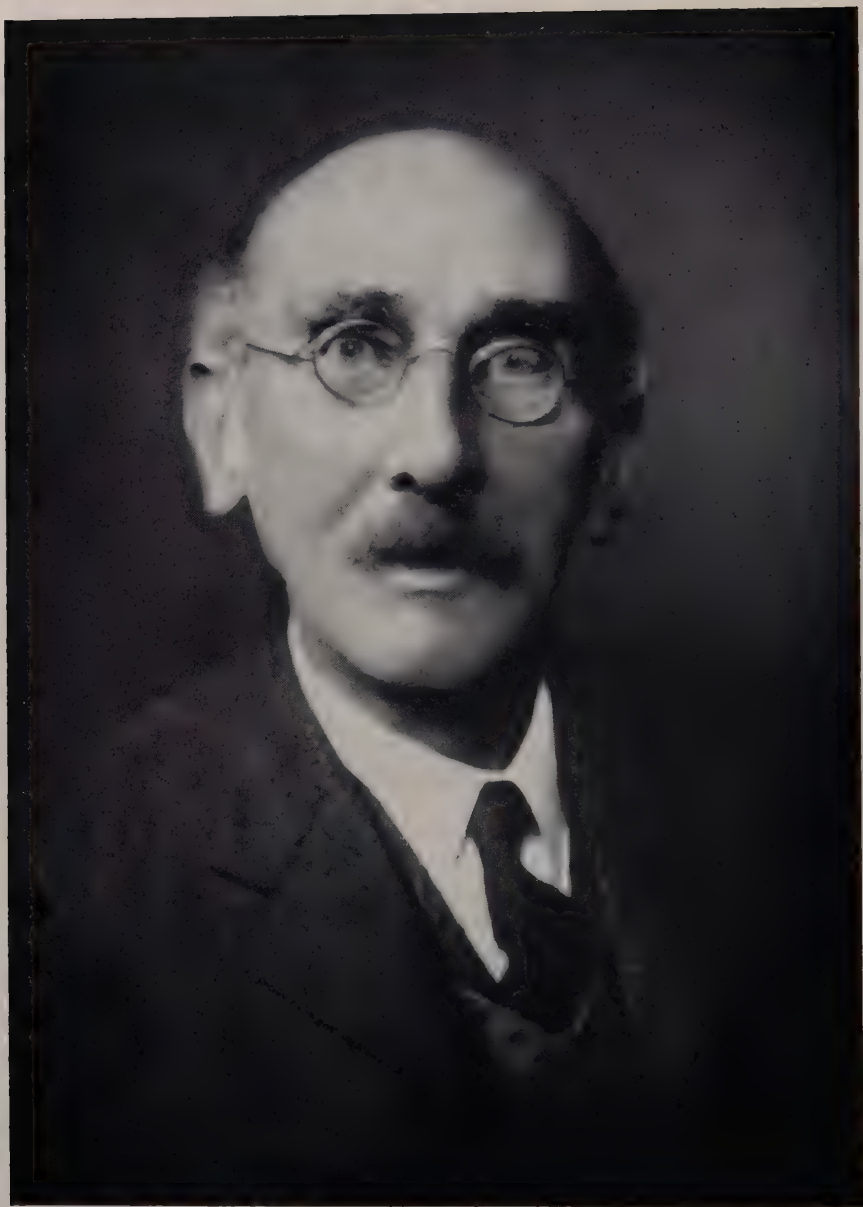
Fig. 1. *Saccharomyces pretoriensis* n.sp. Spores and protuberances in a sporulating culture. ($\times 1000$; sunlight dark-ground microscopy.)

(Received 10 November 1955)



J. P. VAN DER WALT & I. T. TSCHUSCHNER—*SACCHAROMYCES PRETORIENSIS*.
PLATE 1

(Facing p. 486)



CHARLES JAMES MARTIN

Obituary Notice

SIR CHARLES JAMES MARTIN, 1866-1955

Sir Charles Martin, who died on 15 February 1955 in his 90th year at his home at Chesterton, Cambridge, was an honorary member of the Society for General Microbiology. Although primarily a physiologist the interest and influence of this remarkable man extended to all aspects of medical research, including microbiology; in fact it was in this last field of research that Martin did some of his most important work. It is doubly fitting, therefore, that the Society should pay tribute to the memory of this distinguished scientist of such varied gifts. There can be few who are ignorant of his record of achievement, and those who were fortunate enough to have worked with him remember him with esteem and affection.

Charles James Martin was born in London in 1866. He was the son of Josiah Martin, who was an actuary on the staff of a London insurance company. Because the boy Charles was thought to be delicate he was sent to a private boarding school at Hastings, where it was hoped that his health would be improved by living at the seaside. It was intended that he should become an actuary like his father, and on leaving school he spent a year in his father's office. His interests did not lie in this direction, however, but rather in the natural sciences, and after a period of work at Birkbeck College he matriculated and went to King's College, proceeding later to St Thomas's Hospital to study medicine. He had a brilliant undergraduate career. He was an exhibitioner and London University scholar in physiology, and he was awarded a gold medal in this subject. Appointed lecturer in physiology at King's College he continued to hold this post whilst doing his clinical work at St Thomas's, since it was necessary for him to earn if he was to complete his medical studies. He also worked for a time under the famous German physiologist Karl Ludwig at the University of Leipzig, for whom he retained a very high regard.

Martin qualified in medicine in 1889, proceeding to the M.B. in the following year. In 1891 he went to Sydney University as demonstrator in physiology where he replaced Almroth Wright, and the twelve years that he spent in Australia before returning to this country to take over the directorship of the Lister Institute were to prove of the utmost formative value, not only to Martin himself but also to medical teaching and research in Australia. After holding the post at Sydney for six years Martin moved to Melbourne, where he had been appointed to the lectureship in physiology and in 1901, only four years later, he was made professor. As a teacher he was proving himself an outstanding success. His approach to his subject was original and arresting and this, coupled with a clarity of presentation which he possessed in a very high degree, caught the attention of his students and fired them with an enthusiasm approaching his own. Amongst his colleagues at Melbourne were

some who like himself were fine teachers, and together they built up a school of medicine which was to exert a profound influence on the pattern of medical teaching throughout Australia, an influence which continues to this day. And it is not unreasonable to suggest that the rise to eminence in various branches of medicine of so many Australian graduates during the past half century is attributable directly or indirectly to Martin's teaching.

Good though Martin was as a teacher he excelled as an investigator, for he was essentially a man of the laboratory. Skilful technically and delighting in the improvisation of apparatus from bits and pieces for which he had a perfect genius, he asked nothing better than to be at work at the bench on some problem or other. He possessed in full measure that rare gift of being able clearly to discern the essence of a problem and then, having revealed it in all its nakedness, with the deft strokes of the complete artist he would design the experiments calculated to reveal its innermost secrets. Despite the preoccupation of teaching and the administration of growing university departments Martin found time to do quite a lot of original work during the years he spent in Australia; in fact some of his most important researches belong to that part of his career. He investigated the metabolism and heat exchange in marsupials and monotremes, and he made a study of the formation and behaviour of antibodies to snake venom. This latter research was of first-class importance, and in Martin's opinion probably his best piece of pure research, and one imagines that it was largely on the score of this work that he was elected to the fellowship of the Royal Society in 1901 at the early age of 37. In view of the interest of this work to microbiologists it is surprising to find how few are familiar with it. When nearly forty years after its publication certain virus workers were claiming that viral antibodies behaved differently from other antibodies, in that it was possible by dilution or centrifugation readily to recover antibody from apparently neutral mixtures of virus and antibody, it was overlooked that Martin had shown that the same was true of neutral mixtures of a toxin and its antitoxin, though in his work gelatin coated filters were used to separate the two. The truth is that the behaviour of all antibodies is fundamentally similar in that all will unite with the antigen for which they are specific, though the time taken for firm union between the two to occur may vary.

Martin's reputation depended as much on his achievements in the laboratory as his successes in the lecture theatre, he was held in esteem by students and colleagues alike, and in fact he became an almost legendary figure whose influence was to be a lasting inspiration. During the years that he was at the Lister Institute no Australian graduate coming to this country for post-graduate study failed to visit the Lister, if not to work under Martin then at least to pay him homage. And when in 1952 the Australian Commonwealth was celebrating its Jubilee, it paid tribute to Martin by founding in his name two research fellowships in medical science. Mr R. G. Menzies, the Prime Minister, when announcing this sent Martin, who was then in his 86th year, the good wishes of medical research workers throughout Australia, and the National Health and Medical Research Council of Australia sent him a memorial letter, the words of which are worth quoting as evidence of the regard in which

Martin continued to be held: 'Your work and teaching in Australian institutions laid a solid foundation of research in this country and your example and encouragement stimulated its progress through its formative years. Your inspiration still permeates its whole fabric and you are remembered by Australian workers as one of their most distinguished masters.' What a magnificent tribute!

Martin left Australia in 1903 to become director of the Lister Institute, a post which he was to hold until his retirement twenty-seven years later. This institute was formed as the result of the activities of a group of eminent scientists and laymen, who were of the opinion that an institute on the lines of the Pasteur Institute in Paris ought to be set up in this country. After somewhat protracted discussion with the Board of Trade the new venture was incorporated in 1891 as the British Institute of Preventive Medicine, with Lord Lister as its first chairman and Sir William Roscoe as treasurer. Two years later, when sufficient funds had been collected for a start to be made, a search for suitable accommodation was solved, at least temporarily, by amalgamating with the College of State Medicine which had quarters in Great Russell Street, and the Institute opened under the directorship of Dr Armand Ruffer. These quarters were soon to prove inadequate and, a convenient site having been obtained in Chelsea Gardens through the good offices of the Duke of Westminster, a new building was erected and opened in 1897. Since this coincided with the centenary of Jennerian vaccination against smallpox the name of the Institute was changed to the Jenner Institute of Preventive Medicine, a change which was to prove an embarrassment, however, because of confusion with a commercial undertaking making calf lymph which called itself the Jenner Institute. So in 1903, when Lord Iveagh made his munificent gift of a quarter of a million pounds to endow the Institute the name was changed to its present one, and the building as it stands at present was completed in 1910.

When Martin assumed the direction of the Lister Institute it had hardly got under way; it was not a question of taking over a going concern with an established reputation, much required doing, and the subsequent rise to eminence of the Lister Institute as a centre of medical research can without unfairness to anyone be attributed very largely to Martin. No better choice for director could have been made. Arriving fresh from his triumphs in Australia and with a confidence born of experience he set about building the Institute on sound lines. A man of Martin's scientific standing had little difficulty in attracting to his staff men of first-class ability, and with his wide knowledge of the different aspects of medical research in addition to his own speciality of physiology, he was able, as director, to make the fullest use of them. Amongst those who have been members of the Institute's staff the names of Harden, Boycott, Ledingham, Arkwright, Harriette Chick, Muriel Robertson and Robison come to mind, all of whom were to rise to eminence, and with the help of such people the Lister Institute soon came to occupy an important position as a centre of medical research. Young graduates anxious for an opportunity of postgraduate study in one of the medical sciences sought

in increasing numbers the privilege of working there, the majority coming from Britain and the dominions, Australia in particular, though some were from other parts of the world.

Others writing of the Lister Institute under Martin's direction have said what a pleasant and profitable place it was to work in and to this I can add my testimony. I went there in 1913 in the very junior capacity of a research student to work with Ledingham, and it was indeed a happy and stimulating experience. The high standard of Martin's work, his readiness to advise and help, and his enthusiasm set an example which affected even the most junior workers at the Institute. But his success as a director did not depend on this alone. In the scientific world outside he occupied a position of importance, and by serving on the many committees and advisory bodies which he was invited to join, he not only gave invaluable help to medical research but he brought into prominence the institute which he directed. It is not easy for the director of a scientific institute to strike the right balance between these internal duties and external activities, but Martin succeeded admirably; he possessed all the attributes of the ideal director.

Despite his multitudinous duties as director of the Lister Institute Martin found time for research. With Dame Harriette Chick he made a most valuable investigation of the mechanism of disinfection; to this day the Chick-Martin method of testing an antiseptic stands unchallenged. With her he also studied the heat coagulation and precipitation of proteins. And it was his recognition of the importance of Gowland Hopkins's early researches on growth factors which led to the investigation of the accessory food factors, or vitamins as we now know them, at the Lister Institute, and eventually to the formation of the division of nutrition which, under the leadership of Dame Harriette Chick, did such valuable work. In particular, the investigation of rickets in Austria, made in conjunction with the Medical Research Council after the cessation of hostilities in 1919, calls for special mention. But it is the work which Martin did at this period of his career on typhoid and plague which is of most interest to microbiologists. The War Office decided to set up a committee to go into the question of anti-typhoid inoculation which had had a somewhat ineffectual trial in the later stages of the Boer War, Martin being invited to be its Chairman; and he took a large share in planning the work of the committee set up jointly by the India Office, the Royal Society and the Lister Institute to investigate plague in India. These researches on the ecology of plague, establishing the role of the rat flea and the mechanism by which it transmits infection, have become a classic and they owed much of their excellence to Martin. Incidentally this necessitated his spending some time in India, and it was on the outward journey on the S.S. *Caledonia* in 1905 that the famous episode occurred when Martin and three of his fellow passengers, coached by him, beat a team of physical culturists, voyaging to the East, at tug-of-war; a contest which was organized to relieve the tedium of deck games.

The outbreak of hostilities in 1914 saw little immediate change in the work of the Lister. It is true that Martin took immediate steps to increase the output of tetanus antitoxin at the Institute's country division at Elstree, a step

the wisdom of which was not fully appreciated at the time, and one or two of the junior workers left to join one of the services, but apart from this things went on unchanged for the first month or two of war. When, however, the western front became stabilized after the battle of the Marne, and it seemed clear that the allies were in for a long and stubborn war, more and more members of the Institute's staff departed on war service. Martin himself went in 1915, joining the Australian Army Medical Corps with the rank of lieutenant-colonel, and he saw service first of all in Gallipoli. A laboratory service on this front hardly existed at that time and Martin set himself to organize one, and with its aid he did invaluable work on the diagnosis and control of the enteric and dysenteric diseases which were widespread in the troops on the peninsula. Whilst in the Middle East he saw service in Palestine and Egypt, and in 1917 he was moved to France and posted to 25 Stationary Hospital at Rouen, where he had to organize and run the laboratory services of the hospital. Here again he did splendid work, proving himself as ever an inspiration to his colleagues. Amongst those who came under his influence at 25 Stationary was a young R.A.M.C. sergeant R. T. Brain, and it was with Martin's help and encouragement that Brain qualified in medicine after the war; he now occupies an eminent position as a consultant in dermatology. Twice during Martin's war service the value of his work was recognized by his being mentioned in despatches, and after demobilization he was appointed C.M.G.

With the war over the Lister Institute lost no time in getting into its stride again. Martin's own researches at this period were concerned with the question of thermal exchange and heat regulation of the body under varying conditions of work and environment, experiments which he conducted on himself entailing at times standing scantily clad on the roof of the Institute in bitterly cold weather. The Croonian Lectures of the Royal College of Physicians, which Martin delivered in 1930, were devoted to this subject. And in this post-war period he also worked with Robison on the biological value of food proteins, he and his colleagues acting as the experimental animals in much of this work. Of course the mere reference to the scientific publications bearing Martin's name gives a very inadequate idea of the extent of his contribution to medical research. A great deal of excellent work, some of it of first-class importance, was being published from the Institute, and amongst all these researches there was little that did not owe something to the wisdom or inspiration of the Director; his wide knowledge and great skill as an experimenter were always available to his colleagues, and he helped unselfishly without any thought of recognition. Another thing which curtailed the time available for his own researches was the service he gave on outside bodies; skilful as a chairman and a good committee man, his help in one or other capacity was often sought. In addition to the committees on anti-typhoid inoculation and plague already mentioned on which Martin served in the years before the First World War, in that same period he was a member of the Council of the British Medical Association off and on from 1904 to 1916, and he presided over the Section of Pathology at the Annual Meeting of the Association in 1908, and the Section of Bacteriology two years later. Between the two wars he was a member of the Medical

Research Council from 1926 to 1930, and in this capacity as well as a member of many of the Council's committees he gave invaluable service.

When Martin retired from the directorship of the Lister Institute in 1930 he was still, at the age of 65, in full mental and physical vigour, so that when he was invited by the Australian Government to become head of the Division of Animal Nutrition of the Australian Council of Science and Industrial Research to assist with problems connected with that country's health and economy, he readily accepted. And this provided the occasion for Martin's association with yet another of Australia's universities, because the institute that Martin was to direct formed part of the University of Adelaide and the invitation from the Australian Government was coupled with one from the University to occupy their Chair of Biochemistry and General Physiology.

Martin returned to England three years later going to live at Chesterton outside Cambridge, and though in retirement, he continued actively to be engaged on research projects. He made a study of the nutritive value of maize in pigs as part of a larger investigation, made with colleagues at the Lister Institute, of the pellagra problem, but it was his researches on myxomatosis which were probably the most interesting, particularly in the light of recent happenings in this country. His studies of this disease at Cambridge in the years 1934 and 1935 led him to the conclusion that myxomatosis spread from rabbit to rabbit by contact and that spread through the agency of insects was so remote a possibility that it could be ignored. Satisfied on the correctness of this view he felt justified in trying experiments on a larger scale than those which had been possible at Cambridge. So he attempted to establish myxomatosis in the extensive rabbit population of the island of Skokholm off the coast of South Wales, and though he made three attempts to do this between 1936 and 1938 all failed; the inoculated rabbits which he liberated died of the disease without it spreading to any extent to the local rabbits. In this country the devastating spread of myxomatosis amongst the rabbits subsequent to its introduction from France in 1954 has been due apparently to the European rabbit flea *Spilopsyllus cuniculi*, which, as Locksley has recently shown, is absent from the rabbits on Skokholm. It is present, however, on the rabbits of the adjacent island of Skomer and when myxomatosis arrived in the island from the mainland in October 1954 it destroyed the rabbit population.

With the outbreak of the Second World War necessitating the removal of most of the workers and equipment of the Lister Institute to safer quarters than those in Chelsea, Martin invited the division of nutrition to come and work in his large house at Chesterton. Roebuck House, where he had gone to live in 1934 on his return from Adelaide, had been an inn at one time and had ample accommodation in which temporarily to house the displaced workers, and Martin delighted not only in having them as his guests but also in exercising his ingenuity in adapting the rooms and outbuildings to their new use. The five years that the division of nutrition enjoyed hospitality at Roebuck House were a source of real happiness to Martin. When the war ended he was beginning to feel the weight of advancing years—he was in his 79th year—though he was to maintain his interest in scientific things to the end. He continued to act as

chairman of the Committee of the Dunn Nutritional Laboratory until 1946 and to advise the International Wool Secretariat until 1949; his last scientific paper was published in 1948.

Although Martin set no store in honours it was inevitable that many should come his way. Several have already been mentioned, of these his election to the Royal Society and the C.M.G. for his services in the 1914–18 War were the more important. In addition, he was the recipient of the Royal Medal of the Royal Society in 1923, and many Universities conferred on him honorary degrees. He held the honorary doctorate of science of the universities of Sheffield and Trinity College Dublin, the LL.D. of Edinburgh, the D.C.L. of Durham and he received the honorary M.A. of Cambridge. He was elected to the Fellowship of the Royal College of Physicians and he was a Fellow of King's College, London. And in recognition of his services to medical research he was knighted in 1927.

Despite all these honours and his many successes Martin remained simple in his tastes and free from anything in the way of conceit or affectation; he possessed too much common sense for that. He had a very clear sense of right and wrong, and he was fearless in his support of what he held to be right and just regardless of whom he might find opposed to him. Forthright in his approach, at times almost to the point of brusqueness, he had none the less the gift of commanding the devotion of all who worked with him; he was, in fact, a true leader of men. Generous to a degree he would go to endless trouble to help junior colleagues provided they showed a real enthusiasm; even ignorance was sometimes condoned! What Martin could not tolerate was slipshod work, it angered him and excited scathing comment and the bluffer and the pompous received short shrift. But it was his essential kindness that remains most in one's memory. I had returned to the Lister Institute in 1921 as a junior member of the staff and, living nearby and being unmarried then, I often returned to work in my laboratory after the evening meal. And often Martin would look in and take me off to his flat where over cups of tea, which in the absence of Lady Martin he invariably made Australian fashion in a billy-can, we would discuss my research projects. Those were indeed good days!

Martin married Edythe, the daughter of Alfred Cross of Hastings. Lady Martin died in 1954 eleven months before Sir Charles. They leave a daughter.

S. P. BEDSON

The Nature of the Lesion in the Succinate-requiring Mutants of *Neurospora crassa*: Interaction between Carbohydrate and Nitrogen Metabolism

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SUMMARY: Mutants of *Neurospora crassa* requiring dicarboxylic acids for an immediate growth response (*suc* and *at suc*) oxidize acetate, and are inhibited by fluoroacetate with consequent citric acid accumulation to approximately the same extent as the wild-type. The concentration of nitrogen (as ammonium and nitrate salts) present in the conventional growth medium is inhibitory to the growth of these mutants and leads to an accumulation of acetylmethylcarbinol, pyruvic acid and α -ketoisovaleric acid. This inhibition is reduced and growth is stimulated by the addition of dicarboxylic acids or by diminution of the nitrogen present in 'minimal' medium. The addition of nitrogen salts to *suc* mutants probably diverts dicarboxylic acids (already in short supply) from the catalysis of the oxidation of C_2 fragments via the tricarboxylic acid cycle to other reactions. This effect of nitrogen salts upsets the already precarious dicarboxylic acid balance of the *suc* mutants leading to a growth requirement and to the accumulation of intermediates.

This investigation was begun because the existence of mutants of the ascomycete *Neurospora crassa*, which grow on minimal medium only after the addition of small quantities of dicarboxylic acids (succinate-requiring mutants), seemed difficult to reconcile with an apparent use of the tricarboxylic acid cycle for both synthesis and energy by *Neurospora* spp. (Lewis, 1948; Strauss, 1955*a*). Accepting the idea of a 'genetic block' in the cyclic metabolism of dicarboxylic acids by the succinate-requiring mutants seems to require the assumption that acetate is oxidized by some mechanism other than the tricarboxylic acid cycle (Krebs, Gurin & Eggleston, 1952) or that the 'genetic block' is not absolute. Since either of these explanations would be of interest, the properties of the succinate-requiring mutants have been studied in some detail.

It now appears that the succinate-requiring mutants do use the tricarboxylic acid cycle, and that there is no enzymic block to the operation of the cycle. Dicarboxylic acids required to catalyse acetate oxidation are in short supply and, as a result, excess ammonium or nitrate nitrogen inhibits these mutants by withdrawing this limited supply of dicarboxylic acids for synthetic purposes. Inhibition by excess nitrogen is at least partly responsible for the failure of succinate-requiring mutants to grow normally on 'minimal medium' (Beadle & Tatum, 1945).

It is the purpose of this paper to present the evidence for the interaction of carbohydrate and nitrogen metabolism in the succinate-requiring mutants, and to discuss briefly the implications of these findings.

METHODS

The methods used in growing *Neurospora crassa* have been previously described (Strauss & Pierog, 1954; Strauss, 1955*a*). Conidia were prepared by allowing cultures to grow 7 days at room temperature in the light on minimal medium with succinate (0.5 % disodium succinate) and 1.5 % agar added. The conidia were harvested by being suspended in water, filtered through cheese-cloth, washed twice with water by centrifugation and finally suspended in the experimental medium as indicated below. The analytical, enzymic, isotopic and manometric methods used in this investigation were identical with those previously used, except where otherwise indicated.

GENETIC MATERIALS

Two mutations, *suc* and *at*, were used in these studies. The *suc* strains were derived from strain 46005, a succinate-requiring mutant described by Dubes (1953) and obtained from Mrs M. B. Mitchell of the California Institute of Technology, and from strain 86, a double mutant derived as described below from one of the 'glutamate' mutants described by Woodward (1954). Both strain 46005 and Woodward's 'glutamate' mutants are phenotypically similar to the strain described by Lewis (1948) and to strain 46403 (Dubes, 1953; Strauss, 1955*a*); that is they grow readily with either malate, fumarate, succinate or α -ketoglutarate and after a lag (Fig. 1) with acetate and glutamate. The two *suc* strains used (46005 and 86) probably represent independent occurrences of the same *suc* mutation since: (a) heterocaryons growing on minimal medium are not formed by the two strains; (b) wild-type recombinants were not obtained from among 295 single ascospore cultures picked from a cross of 46005 \times 86. The probability of not detecting a wild-type recombinant from this number of random segregants from a cross of non-allelic genes is less than 1 %, even if the genes are as close together as 0.8 map unit. Perithecia are formed only sparsely in this cross, and most of the resulting ascospores do not acquire the black coat characteristic of ripe spores. For our tests, spores were plated on Petri plates containing solid minimal or minimal plus succinate medium, the spores were activated and the plates were incubated at 25°. Only spores which had germinated within 15 hr. incubation were picked for testing.

In this paper the succinate-requiring mutants are designated *suc* with the wild-type allele designated *suc*⁺, the symbolism used by Barratt, Newmeyer, Perkins & Garnjobst (1954). We have used absence of growth in minimal medium and response to both succinate and acetate (tested separately) as growth supplements after 3 days of incubation as a diagnostic test for the *suc* phenotype. The evidence that *suc* represents a single gene mutation is given in Table 1. Of the total of 256 ascospores tested from the type cross *suc* \times *suc*⁺, 130 were *suc*⁺, 126 *suc*, a close fit to the expected 1:1 ratio.

Strain 86 was obtained by the method of Woodward, De Zeeuw & Srb (1954), starting with a 'glutamic' mutant obtained by Dr Woodward from wild-type

74A by X-irradiation. Further X-irradiation of the conidia of this mutant followed by incubation with shaking in minimal medium plus glutamate resulted after repeated filtration in the isolation of a strain which differed phenotypically from the original. This new strain (86), obtained in the course of work at Brookhaven National Laboratory, did not grow within 3 days on either acetate or glutamate supplements, although it still responded to either

Table 1

Parental strains	Presumed parental genotypes	Phenotypes of segregants	Total segregants		No. of classifiable asci for gene indicated	Percentage second division segregation
			Random	From ordered asci		
46005 \times 8a	<i>at⁺ suc</i>	<i>at⁺ suc</i>	—	31	18 (<i>suc</i>)	11
	\times					
	<i>at⁺ suc⁺</i>	<i>at⁺ suc⁺</i>	—	43		
15300 \times 46005	<i>alb at⁺ suc⁺</i>	<i>alb at⁺ suc⁺</i>	21	30	12 (<i>alb</i>)	50
	\times	<i>alb⁺ at⁺ suc</i>	32	23		
	<i>alb⁺ at⁺ suc</i>	<i>alb at⁺ suc</i>	8	32	12 (<i>suc</i>)	42
		<i>alb⁺ at⁺ suc⁺</i>	16	20		
86 \times 15300	<i>alb⁺ at suc</i>	<i>alb⁺ at⁺ suc⁺*</i>	—	30	16 (<i>alb</i>)	75
	\times	<i>alb at⁺ suc⁺*</i>	—	70		
	<i>alb at⁺ suc⁺</i>	<i>alb at suc</i>	—	16	16 (<i>suc</i>)	16
		<i>alb at⁺ suc</i>	—	33		
		<i>alb⁺ at suc</i>	—	22	8 (<i>at</i>)	0
		<i>alb⁺ at⁺ suc</i>	—	42		
86 \times 5297	<i>at suc</i>	<i>at⁺ suc⁺*</i>	—	27	4 (<i>suc</i>)	0
	\times	<i>at⁺ suc</i>	—	14		
	<i>at⁺ suc⁺</i>	<i>at suc</i>	—	7	4 (<i>at</i>)	0
27-1† \times 46005	<i>at suc⁺</i>	<i>at⁺ suc⁺*</i>	—	36	10 (<i>suc</i>)	0
	\times	<i>at⁺ suc</i>	—	23		
	<i>at⁺ suc</i>	<i>at suc</i>	—	18	10 (<i>at</i>)	0

Phenotypes: *alb*=albino; colourless mycelium. *alb⁺*=wild type coloration. *at⁺ suc*=*suc*=no growth in 3 days on minimal medium; growth with succinate or acetate added as supplements. *at suc*=no growth in 3 days on minimal medium or on minimal + acetate; growth on minimal + succinate. *at⁺ suc⁺*=wild type=growth on minimal medium.

* The *at⁺ suc⁺* phenotype includes the class of *at suc⁺* strains since these grow on minimal medium (see Table 2).

† See Table 2 for the origin of this strain.

succinate, fumarate, malate or α -ketoglutarate. The new strain obtained was crossed to wild-type and a high conidiating segregant (86-1) was obtained. The evidence that strain 86-1 is a double mutant is summarized in Table 1. Crosses of 86-1 to wild-type or to the morphological mutant, albino, give asci-yielding nutritional mutants of parent type (no growth on acetate) and recombinants (mutants growing on acetate) with spore pairs always giving the same results. Furthermore, a supposed *at suc⁺* strain selected from ascus #27 of the cross 86 \times 5297 (Table 2), when crossed to strain 46005 (supposed *at⁺ suc*) gave asci containing spores yielding cultures with *at suc* phenotypes, as would be expected if this cross (27-1 \times 46005) were *at suc⁺ \times at⁺ suc*. On totalling the data in Table 1 it will be noted that a total of 112 *at⁺ suc* strains were obtained

from the type cross *at suc* × *at⁺ suc* compared to a total of 63 *at suc*. We believe that this discrepancy from the expected 1:1 ratio probably is due to the relatively low percentage germination of *at suc* ascospores compared to *at⁺ suc* spores for which we have other evidence. No classifiable ascus has been obtained that is not in accord with the hypothesis of a single modifier gene. This gene was designated *at* to indicate its effect on the utilization of acetate, and to distinguish it from the acetate requiring mutants described earlier under the designation *ac* (Strauss & Pierog, 1954). The *at* locus apparently acts only as a modifier to the *suc* gene, preventing response of *suc* strains to acetate. In the presence of the wild-type allele of *suc*, the *at* gene apparently has no effect since strain 27-1 (*at suc⁺*) grows normally on minimal medium.

Table 2. *Composition of ascus 27*

	Parents	...	86-1 × 15300	
	Growth response of parents			
	Minimal		0	+
	Minimal + succinate		+	+
	Minimal + acetate		0	+
	Presumed genotype of parents		<i>alb⁺ at suc</i>	<i>alb at⁺ suc⁺</i>
	Segregants spore pair number			
Growth	1	2	3	4
Minimal	+	+	0	0
Succinate	+	+	+	+
Acetate	+	+	+	+
Colour	Normal	Albino	Normal	Albino
Presumed genotype	<i>alb⁺ at suc⁺</i>	<i>alb at suc⁺</i>	<i>alb⁺ at⁺ suc</i>	<i>alb at⁺ suc</i>

RESULTS

Growth studies

Both *suc* and *at suc* strains grow well in the presence of succinate (Figs. 1, 2), *suc* grows on acetate and on glutamate after a short lag, while the *at suc* strain grows on acetate only after a considerable lag period. At about the same time that *at suc* strains show visible growth with an acetate supplement, *suc* strains initiate growth on minimal, and the growth of *suc* on minimal roughly parallels the growth of *at suc* on acetate. The *suc* mutants are not permanently adapted after initiation of growth on minimal medium since subinoculations of cultures that are growing on minimal medium on to fresh minimal medium require a long lag period before again starting to grow. As can be seen from the scatter of points in Fig. 1, the time at which cultures of *suc* on minimal medium or of *at suc* on acetate begin to grow rapidly varies noticeably from flask to flask.

When cultures are grown with varying amounts of nitrogen (added as ammonium sulphate and substituting equimolar amounts of sodium potassium tartrate for the ammonium tartrate usually present) there is an inhibition of mutant growth by quantities of nitrogen that do not inhibit the wild-type (Fig. 3). This inhibition is counteracted by the addition of succinate (Table 3).

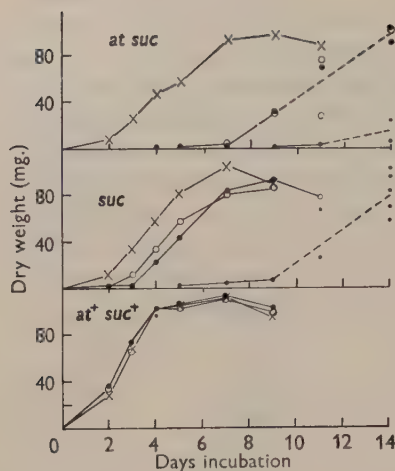


Fig. 1

Fig. 1. Growth of *suc* mutants as a function of time. Additions to 20 ml. minimal medium: 25 μ M succinic acid or glutamic acid as sodium salts. 50 μ M acetic acid as the sodium salt: x, succinate added; O, acetate added; ●, glutamate added; •, no addition.

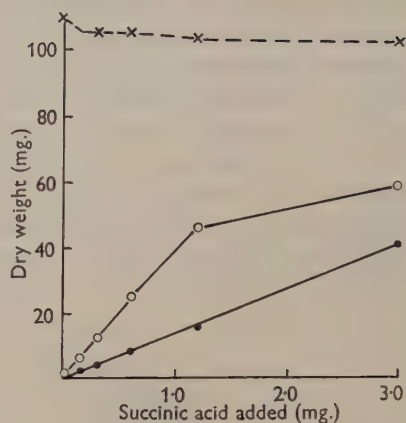


Fig. 2

Fig. 2. Growth of *at suc* and *suc* strains as a function of succinate concentration. Growth after 4 days at 28°. Succinic acid added as disodium salt: x, 7A (*at+ suc+*); O, 46005 (*suc*); ●, 86 (*at suc*).

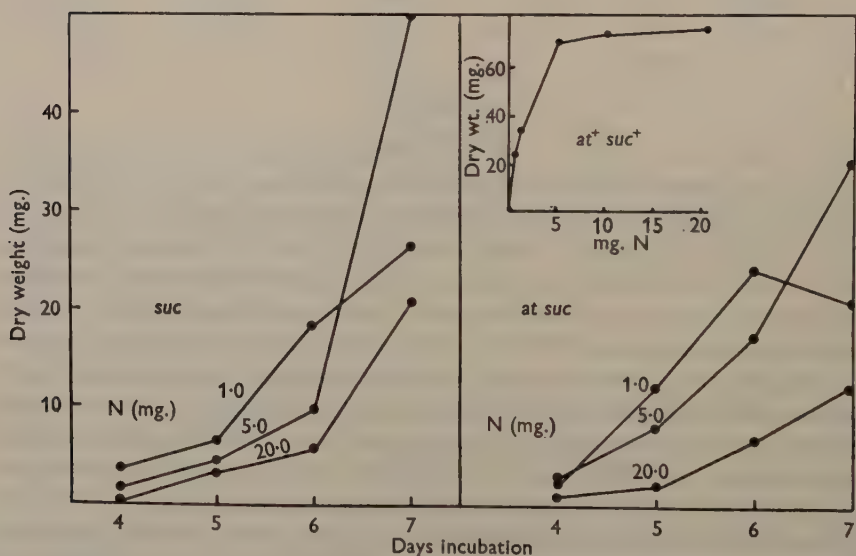


Fig. 3. Effect of nitrogen concentration on growth of *suc*. Wild-type curve of growth as a function of nitrogen concentration obtained after 3 days' incubation. *suc* grown with no organic supplement. *at suc* grown with 5 mg. $\text{Na}_2\text{C}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ per 20 ml. Nitrogen added as $(\text{NH}_4)_2\text{SO}_4$.

Both *suc* and *at suc* strains are inhibited by the amount of nitrogen ordinarily present in minimal medium (22.2 mg./20 ml. as ammonium tartrate and ammonium nitrate).

Accumulations

The *suc* strains accumulate acetylmethylcarbinol (AMC, 3-hydroxy-2-butanone), pyruvic acid and α -ketoisovaleric acid when grown on limiting succinate concentrations. AMC was first recognized by its characteristic odour and by the Voges-Proskauer reaction (Westerfeld, 1945) given by steam distillates of culture medium. It was identified in the steam distillate from *suc* culture medium by the formation of the characteristic precipitate of nickel dimethylglyoxime after oxidation (of the steam distillate) with FeCl_3 in acid, with subsequent distillation of the oxidation product into neutralized hydroxylamine in the presence of nickel chloride and sodium acetate. This compound (AMC) has been previously identified as an accumulation product of neurospora mutants requiring acetate for growth (Strauss, 1953).

Table 3. *Effect of succinic acid on the inhibition of the growth of a suc strain by ammonium nitrogen*

Succinic acid (mg./flask)	mg. N per flask					Strain
	0.5	1.0	2.0	8.0	25.0	
0	12	13	9	4	2	<i>suc</i> (46005)
0.6	22	31	37	30	21	
1.2	25	39	45	41	42	
0	22	35	50	105	103	<i>suc</i> ⁺ (7A)

Recorded growth as mg. dry wt. after 4 days at 25° in medium using sodium potassium-tartrate. Nitrogen added as ammonium sulphate. Succinic acid added as disodium succinate. Values averages of duplicates.

Accumulation of keto acids in culture media in which *suc* strains had grown was indicated by the formation of 2,4-dinitrophenylhydrazine derivatives which could be extracted from ethyl acetate by sodium carbonate and then returned to an ethyl acetate layer after acidification. Pyruvic acid was identified by chromatography as one of the accumulation products of the dinitrophenylhydrazones after two passages through sodium carbonate; a spot coincided with that given by an authentic sample of pyruvic 2,4-dinitrophenylhydrazone after ascending chromatography in tertiary amyl alcohol : ethanol : water (50 : 10 : 40) or in isobutanol : water (90 : 10). Another major spot (distinct from the second spot given by the pyruvic acid derivatives) was noted with an R_f of 0.83 in the tertiary amyl alcohol mixture and with an R_f of 0.5 in isobutanol : water. These R_f values, higher than those characteristic of pyruvic acid, indicate a compound with more non-polar character than pyruvic acid. We have provisionally identified this compound as the derivative of α -ketoisovaleric acid since the unknown compound gave a spot which was superimposed on the spot given by the 2,4-dinitrophenylhydrazine derivative of an authentic sample of α -ketoisovaleric acid (kindly

provided by Dr H. E. Umbarger) after chromatography with either the tertiary amyl alcohol mixture or with isobutanol : water.

Cultures of the *suc* strain grown in the presence of succinate show a steadily decreasing accumulation of AMC as growth proceeds (Fig. 4). In the absence of succinate relatively more AMC is accumulated up to the point where rapid growth begins. Nitrogen (added as ammonium sulphate) leads to an increase in the amount of AMC accumulated per mg. dry weight (Table 4), whereas succinate reduces the amount of accumulation.

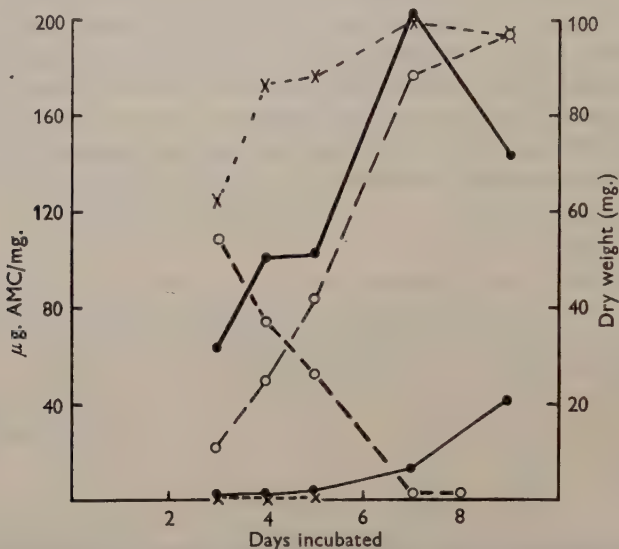


Fig. 4. Amount of acetylmethylcarbinol accumulated by *suc* cultures as a function of time and the presence of succinic acid. Succinic acid = 1.2 mg./flask added as disodium succinate. Lighter lines show dry weight produced. ●—●, *suc* no succinate; ○---○, *suc* plus succinate; ×---×, 7A (*suc*⁺) no succinate.

Table 4. Effect of succinic acid and ammonium nitrogen on the accumulation of acetylmethylcarbinol by a *suc* mutant

Succinic acid (mg./flask)	mg. N per flask			
	1.0	5.0	10.0	20.0
0	329 (4)	—	—	—
0.3	109 (19)	179 (20)	183 (14)	224 (12)
1.2	15 (33)	—	—	—
3.0	1 (33)	—	—	—

Cultures grown 4 days in medium containing sodium potassium tartrate as a substitute for ammonium tartrate.

Nitrogen added as ammonium sulphate. Succinic acid added as disodium succinate.

AMC values as µg. accumulated/mg. dry weight. Values averages of duplicate flasks. Dry weight given in parentheses.

There is a reciprocal relationship between AMC accumulation and germination of the *suc* and *at suc* mutants. Conidia incubated in phosphate buffer with sucrose send out long germ tubes, and if the conidial suspension is dense enough there is visible growth after a 23 hr. shaking period. Nitrogen salts completely inhibit the germination of *at suc* conidia, greatly reduce the germination of *suc* conidia, and cause the accumulation of AMC by both *suc* and *at suc* mutants

Table 5. *Effects of the constituents of minimal medium on germination and acetylmethylcarbinol accumulation*

Addition	Strain 7A (<i>at⁺ suc⁺</i>)		Strain 46005 (<i>at⁺ suc</i>)		Strain 86 (<i>at suc</i>)	
	AMC	Germination	AMC	Germination	AMC	Germination
None	0	Small clumps of mycelium	0	Germination with long germ tubes. Some clumps	0	Good germination with long germ tubes
0.5 % ammonium tartrate	0	Germination but no clumps	38	Some germination but many non-germinated conidia	19	Practically no germination of conidia
0.05 % Mg SO ₄ .7H ₂ O	0	Clumps of mycelium	0	Heavy mycelial clumps formed	0.5	Germination with much mycelial development
0.01 % NaCl, 0.01 % CaCl ₂ trace elements and biotin*	0	Clumps of mycelium	0	Very heavy clumps of mycelium formed	0	Clumps of mycelium
0.5 % KNO ₃	0	Germination with small germ tubes and no clumps	25	Good germination but some non-germinated conidia	20	No germination

AMC as total μ g. accumulated after 23 hr. shaking at 28° determined by direct analysis of the medium after removal of conidia by centrifugation.

Added 4.7×10^7 *suc*, 1.1×10^8 *at suc* and 9.8×10^7 *at⁺ suc⁺* (7A) conidia to 54 ml. of 0.067 M-phosphate buffer pH 6.0 plus 2 % sucrose plus additions as shown. The conidia were harvested from 7-day cultures and washed three times with water by centrifugation before use.

* Trace elements and biotin added as in minimal medium.

(Table 5). Conidia of the *suc* and *at suc* strains suspended in minimal medium and shaken 2–4 hr. in manometer vessels at 30° accumulate measurable amounts of AMC. No accumulation is noted when conidia of these strains are shaken under identical conditions with 2 % sucrose in phosphate buffer (0.067 M, pH 6.0), although sucrose in phosphate buffer is rapidly oxidized by both *suc* and *at suc* strains.

Oxidation studies

The wild-type, *suc* and *at suc* strains oxidize sucrose and acetate readily with no appreciable time lag (Fig. 5). There are no appreciable differences in the rate of mutant and wild-type oxidation. Sodium fluoroacetate inhibits acetate

oxidation by both wild-type and mutant strains (Table 6), with a concomitant increase in the accumulation of citrate (Table 7) as determined by the method of Ettinger, Goldbaum & Smith (1952). Although the data are given for conidia (because of the greater uniformity of this material) *suc* and *at suc* oxidation of acetate and sucrose, inhibition by fluoroacetate and accumulation of citrate can be readily demonstrated with mycelium from 24 hr. shake cultures.

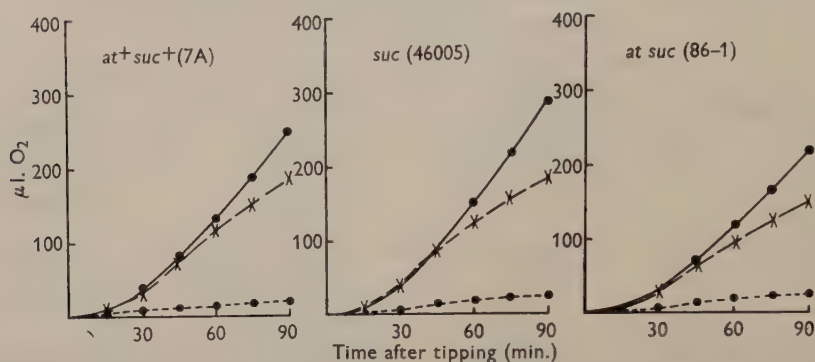


Fig. 5. Oxidation of acetate and sucrose by *suc* and *at suc*. Oxygen consumption measured at 30°. Reaction mixture contained 2 ml. conidial suspension + 0.3 ml. substrate in 0.067 M-KH₂PO₄-Na₂HPO₄ buffer, pH 6.0. ●---●, endogenous; ●—●, acetate, ×—×, sucrose.

Table 6. Inhibition of acetate oxidation by sodium fluoroacetate

Strain	Addition			
	None	Sodium acetate, 10 μM	Sodium fluoroacetate, 20 μM	Sodium acetate plus sodium fluoroacetate
<i>at suc</i>	24	202	8	25
<i>suc</i>	28	233	9	29
7A (<i>at+ suc+</i>)	17	194	5	20

Recorded μl. O₂ taken up in the first 90 min. after tipping. Final volume in the main compartment = 2.5 ml.

Added suspensions of conidia grown 7 days and washed twice with water, once with buffer after harvesting. Suspensions made up to approximately the same turbidity in 0.067 M-phosphate buffer pH 6.0. Two ml. of conidial suspensions added to each vessel.

It is possible to demonstrate an active isocitric-dehydrogenase and glutamic-dehydrogenase in extracts of *suc* and *at suc* prepared and tested by a method similar to that used by Fincham (1954). Extracts of *at suc* prepared from material grown 2 days in shake culture on minimal medium plus succinate contain an active glutamic-dehydrogenase, even though these strains do not show significant amounts of growth with glutamate as a growth supplement before the end of a 7-day lag period (Fig. 6). α -Ketoglutaric acid, sterilized by filtration, produces a growth response by *at suc* as rapidly as does succinate. Although glutamate does not initiate growth within the first 3 days, it does

stimulate growth in this time period in the presence of a limiting amount of succinate (Table 8).

Yemm & Folkes (1954) showed that nitrogen-deficient cultures of *Torulopsis utilis*, suspended under aerobic conditions in a carbohydrate-free medium, doubled or tripled their rate of oxygen uptake on the addition of ammonium nitrogen. Since this appeared to be a method of studying the systems of

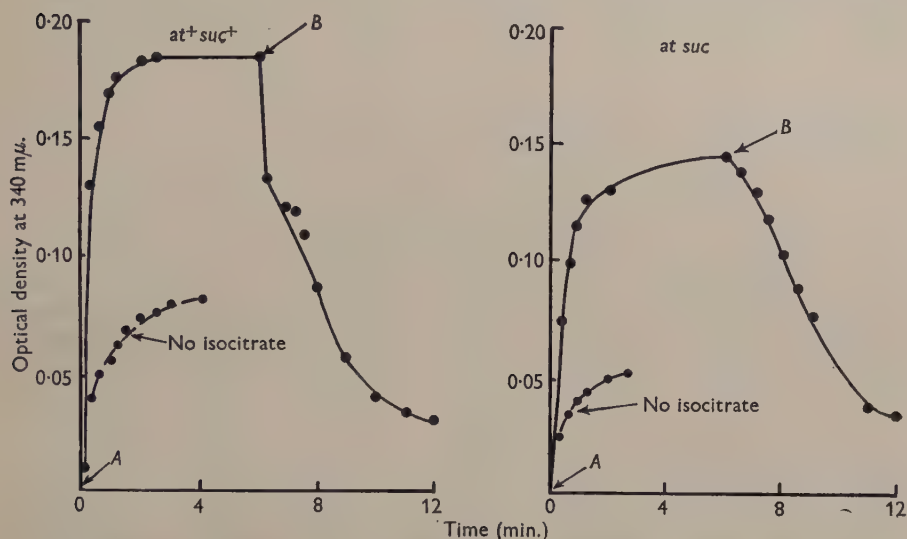


Fig. 6. Presence of glutamic dehydrogenase in an *at suc* strain. Reaction mixtures: isocitrate 400 $\mu\text{g.}$, 0.6 ml. 0.05 M-tris(hydroxymethyl)aminomethane buffer pH 7.2, 0.1 ml. 0.02 M-MnCl₂, 50 $\mu\text{g.}$ TPN (Sigma Chemical Company), 0.1 ml. enzyme extract. Total volume=3 ml. Dotted line without isocitrate. Added TPN at arrow A. At arrow B added 5 $\mu\text{M-}\alpha$ -ketoglutarate, 10 $\mu\text{M-NH}_4\text{Cl}$. Enzyme extracted from 2-day shake cultures grown in minimal medium with succinate, collected, washed, ground with alumina in 0.05 M-KH₂PO₄-Na₂HPO₄ buffer pH 7.2 and centrifuged in the cold at 11,000 g for 20 min. Supernatant used as enzyme preparation.

Table 7. Accumulation of citric acid in the presence of sodium fluoroacetate

Strain	Addition			
	None	Sodium acetate, 100 μM	Sodium fluoro- acetate, 50 μM	Acetate plus fluoroacetate
<i>suc</i> (46005)	64	98	93	160
	—	89	120	190
<i>suc+</i> (7A)	32	57	110	170
	40	69	92	130

Recorded total $\mu\text{g.}$ of citric acid accumulated in a total volume of 2.5 ml. Added 3.4×10^8 *suc+* conidia, 3.7×10^8 *suc* conidia. Citric acid extracted and reaction stopped after two hours' incubation at 30° with shaking by heating in a boiling water bath with 0.5 ml. 9 N-H₂SO₄ and diluting to 10 ml.

carbohydrate metabolism under conditions of maximum utilization, an experiment similar to, but not identical with, that of Yemm & Folkes was performed. Conidia of the various strains were prepared as usual and washed

Table 8. *Stimulation of growth of at suc by glutamate in the presence of limiting concentrations of succinate*

Disodium succinate (mg./flask)	Monosodium glutamate (mg./flask)				
	0	1	2	4	8
0	0	0	0	0.2	0.8
1	5	7	8	10	25
2	13	10	18	26	39
4	21	28	32	37	46
8	32	43	43	46	54

Recorded mg. dry weight produced by *at suc* (86-1A) after 3 days in minimal medium plus additions as shown. All values averages of duplicate determinations.

Table 9. *Effect of nitrogen on oxygen uptake by suc conidia incubated with sucrose*

Expt. no.	Preincubation period (hr.)	Nitrogen	Strain			Time after tipping
			<i>at⁺ suc⁺</i> (7A)	<i>suc</i> (46005)	<i>at suc</i> (86)	
1	1	0	351	239	374	105 min.
			359	232	375	
		+	450	172	284	
			445	182	287	
	24	0	130	246	137	120 min.
			114	183	145	
		+	201	247	144	
			176	210	171	
2	1	0	318	251	257	120 min.
			365	244	—	
		+	533	211	184	
			531	215	169	
	24	0	136	65	136	120 min.
			135	54	134	
		+	254	74	208	
			248	73	181	

Conidia grown 7 days. Harvested and washed twice with water by centrifugation. Suspended in buffer as described in text.

2 ml. suspension added to flask. Side arm contained either 0.2 ml. of water or 0.2 ml. of a solution containing 2.75 mg. N/ml. (as $(\text{NH}_4)_2\text{SO}_4$) as indicated. 0.067 M- KH_2PO_4 - Na_2HPO_4 buffer pH 6.0, 2% sucrose plus 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Recorded total $\mu\text{l. O}_2$ taken up in the indicated time interval.

with 0.067 M-phosphate buffer pH 6.0 containing 2% sucrose and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. One set of conidia of each strain was suspended in this buffer 1 hr., and after resuspending in fresh buffer of identical composition, 2 ml. of this conidial suspension were placed in a manometric vessel containing ammonium sulphate in the side arm (Table 7). The second set of conidia was shaken for 24 hr. in the sugar-containing buffer, then the germinated conidia were strained through cheese-cloth, resuspended in fresh sugar-containing buffer and treated in a manner similar to the conidia of set 1 (Table 9). In these experiments the turbidity of the conidial suspensions of set 1 was

adjusted so that approximately the same number of wild-type, *suc* and *at suc*, conidia were added to the manometers. The suspensions after shaking for 24 hr. were not adjusted in this manner.

The addition of ammonium nitrogen to a 'fresh' conidial suspension results in a stimulation of oxygen uptake by wild-type conidia in a manner reminiscent of the Yemm & Folkes phenomenon, but the addition of ammonium nitrogen depresses the uptake of oxygen by mutant conidia. After 24 hr. shaking in the absence of nitrogen, however, even mutant conidia are slightly stimulated by the addition of nitrogen, although not as much as the wild-type.

Carbon dioxide fixation

The different effect of ammonium on the oxygen uptake of *suc* and *suc*⁺ prompted us to look for the mechanisms that might supply dicarboxylic acids for interaction with ammonium. Since carbon dioxide fixation is one mechanism for the *net* synthesis of dicarboxylic acids (Utter & Wood, 1951) it was decided to measure the amount of carbon dioxide fixation by wild-type and *suc* strains. Conidia prepared by the methods used to study the effect of nitrogen on oxygen uptake (cf. above) were incubated with 0.2 μ c. $\text{NaH}^{14}\text{CO}_3$ in closed manometer vessels in the presence and absence of ammonium nitrogen. At the conclusion of a 2 hr. shaking period the conidia were harvested by centrifugation, washed twice with unlabelled 0.1 M- NaHCO_3 and twice with hot water. The conidial suspensions were then transferred to stainless steel planchets, evaporated to dryness, weighed, and their radioactivity determined in a flow counter operated in the Geiger region. Heat inactivated conidia treated in this way gave a count that was never significantly above the background. An attempt was made to use suspensions containing the same number of wild-type and mutant conidia, and in all cases where absolute comparison was desired haemocytometer counts of the suspensions were made.

The addition of nitrogen to a washed conidial suspension causes a large increase in the amount of carbon dioxide fixed by the wild-type, but has only a small effect on carbon dioxide fixation by the *suc* or *at suc* strains (Table 10). As little as 0.1 mg. of N in 2.6 ml. is clearly effective in increasing the carbon dioxide uptake by the wild-type (Fig. 7). The uptake of carbon dioxide by the wild-type strain is linear in the absence of nitrogen but more irregular in its presence (Fig. 8). Under our conditions the fixation of carbon dioxide is not a linear function of the number of conidia; the ratio of carbon dioxide fixation in the presence and absence of nitrogen is higher when the conidial concentration is low than when this concentration is high.

It was suggested to the author by Dr R. C. Fuller that a significant portion of the total fixed carbon dioxide would be removed by the vigorous washing of the conidia involved in our experiments. We therefore studied the *total* amount of carbon dioxide fixed by stopping the reaction at the conclusion of the incubation period by the addition of trichloroacetic acid (final concentration 10 %, w/v), and then separating the conidia from the acid soluble fraction by centrifugation after 30 min. at 40–50°. Samples of both fractions were

evaporated to dryness, weighed and their radioactivity determined. The counts recorded in Table 11 are corrected for self-absorption.

The wild-type fixes about as much carbon dioxide into conidia as into the acid soluble fraction, both in the presence and absence of an ammonium salt.

Table 10. *Effect of ammonium nitrogen on the fixation of $\text{NaH}^{14}\text{CO}_3$*

Expt.	Nitrogen	Strain		
		7 A (<i>at⁺ suc⁺</i>)	46005 (<i>suc</i>)	86-1 (<i>at suc</i>)
1	0	384	163	369
		396	194	—
	+	2120	217	362
		1650	227	231
		(5.2×10^7)	(2.6×10^7)	(4.3×10^7)
2	0	257	—	168
		425	—	160
		323	—	109
	+	1785	—	245
		1910	—	245
		1890	—	158
		(6.6×10^7)	—	(4.4×10^7)

Recorded total counts per minute after 2 hr. shaking in 2 ml. 0.067 M- KH_2PO_4 - Na_2HPO_4 buffer pH 6.0 with 2 % sucrose and 0.05 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ plus 0.2 ml. $\text{NaH}^{14}\text{CO}_3$ solution containing 1 μC /25 mg. NaHCO_3 /ml.

Values in parentheses indicate number of conidia added (haemocytometer counts). Experiment 1, final washings with hot water. Expt. 2, final washings with hot ethanol.

Manometer vessels shaken 2 hr. at 30° after tipping $\text{NaH}^{14}\text{CO}_3$.

Expt. 1, 6-day-old cultures; heat inactivated conidia showed no incorporation. Expt. 2, 7-day-old cultures; heat inactivated conidia showed no incorporation.

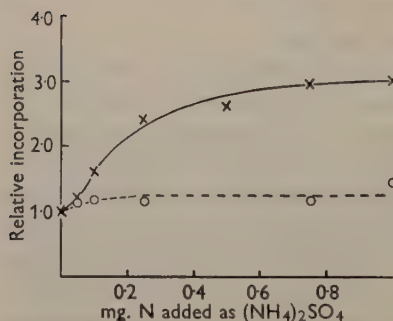


Fig. 7

Fig. 7. Effect of ammonium sulphate nitrogen on the incorporation of $\text{NaH}^{14}\text{CO}_3$ by wild-type and *suc* conidia. Measured incorporation of $\text{NaH}^{14}\text{CO}_3$ as total c.p.m. after 2 hr. Ammonium sulphate added to 2.0 ml. conidial suspension in buffer (0.067 M- KH_2PO_4 - Na_2HPO_4 pH 6.0 + 2 % sucrose + 0.05 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) plus 0.2 ml. $\text{NaH}^{14}\text{CO}_3$ containing 1 μC /25 mg. NaHCO_3 /ml. \times , *suc⁺*; \circ , *suc*.

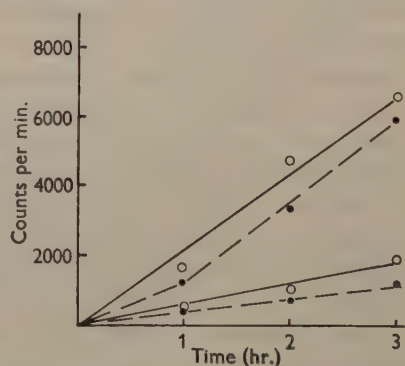


Fig. 8

Fig. 8. Incorporation of $\text{NaH}^{14}\text{CO}_3$ by wild-type conidia as a function of time, conidial concentration and the presence of nitrogen. Dotted curve, 1 ml. conidial suspension; solid curve, 2 ml. conidial suspension. Top curve of each in the presence of 0.5 mg. of nitrogen as $(\text{NH}_4)_2\text{SO}_4$.

The striking feature of this experiment, however, is the effect of ammonium salts in increasing the amount of carbon dioxide fixed into the acid soluble fraction of the mutant tested without causing a concomitant increase in the conidial fraction. More recent experiments indicate that the fixed material in the acid soluble fraction is not ether extractable. Succinate, a compound which will permit growth of the *suc* strains in minimal medium, does not lead to an increase in the amount of carbon dioxide fixed into the conidial fraction, even after 2 hr. preincubation of conidia in succinate-containing buffer.*

Table 11. *Distribution of the $^{14}\text{CO}_2$ fixed by wild-type and at suc conidia*

Fraction	Strain					
	7 A (at ⁺ <i>suc</i> ⁺)		86-1 (at <i>suc</i>)		86-1 (at <i>suc</i>) pre-	
	Nitrogen		Nitrogen		incubated with	
	0	+	0	+	succinate	Nitrogen
Conidia	1720	4900	785	655	801	720
	1670	5280	710	711	819	712
Supernatant (acid-soluble)	2230	6880	520	1610	570	2360
	1960	5280	545	1800	545	2480

Recorded total counts per minute in the various fractions after 2 hr. shaking in 2 ml. 0.067 M-KH₂PO₄-Na₂HPO₄ buffer pH 6.0 with 1 % sucrose and 0.05 % MgSO₄·7H₂O plus 0.2 ml. NaH¹⁴CO₃ solution containing 2 μc . ¹⁴C/25 mg. NaHCO₃/ml.

Nitrogen added=0.25 ml. of a solution of (NH₄)₂SO₄ containing 2 mg. N/ml. Succinate added to buffer where indicated=0.1 % disodium succinate. The conidial concentrations were different for the three strains tested.

DISCUSSION

The unstable balance (Strauss, 1955*b*) between carbohydrate and nitrogen metabolism in the *suc* mutants is seen: (a) in the inhibitory effect of nitrogen salts on the germination and growth of *suc* and *at suc* conidia; (b) in the relationship between the accumulation of AMC and the presence of ammonium salts; (c) in the lack of increased oxygen uptake in the presence of ammonium salts by mutant conidia compared to the wild-type; and (d) by the relative accumulation of acid-soluble products of carbon dioxide fixation in the presence of ammonium salts by the mutant tested compared to the wild-type. Assuming that the supply of dicarboxylic acid is limiting in the *suc* mutants (an assumption required by the growth-promoting effects of dicarboxylic acids), addition of nitrogen salts might be expected to compete with C₂ fragments for the limited supply of C₄ compounds available by forming amino acids or other synthetic products, the nature of which has not yet been established (Fig. 8). Ammonium salts are known to be ketogenic when added to liver slices, and a similar competition for dicarboxylic acids has been suggested by Recknagel & Potter (1951) to account for this ketogenic effect. In neurospora, when the

* L-asparagine increases the amount of C¹⁴O₂ fixed by mutant conidia to almost the wild-type level.

supply of C_4 acids required for C_2 oxidation is diminished, products such as pyruvic acid α -ketoisovaleric acid and AMC accumulate (Fig. 9).

Notwithstanding the growth-promoting action of intermediate compounds of the tricarboxylic acid cycle, it seems likely that the *suc* and *at suc* strains make the same use of the tricarboxylic acid cycle for acetate oxidation as does the wild-type (Strauss, 1955a). Both *suc* and *at suc* strains oxidize acetate as well as the wild-type, even though the *at suc* strain does not start growth with acetate as a supplement to minimal medium until after a 7-day lag period. Fluoroacetate, which is commonly used as an inhibitor of the tricarboxylic

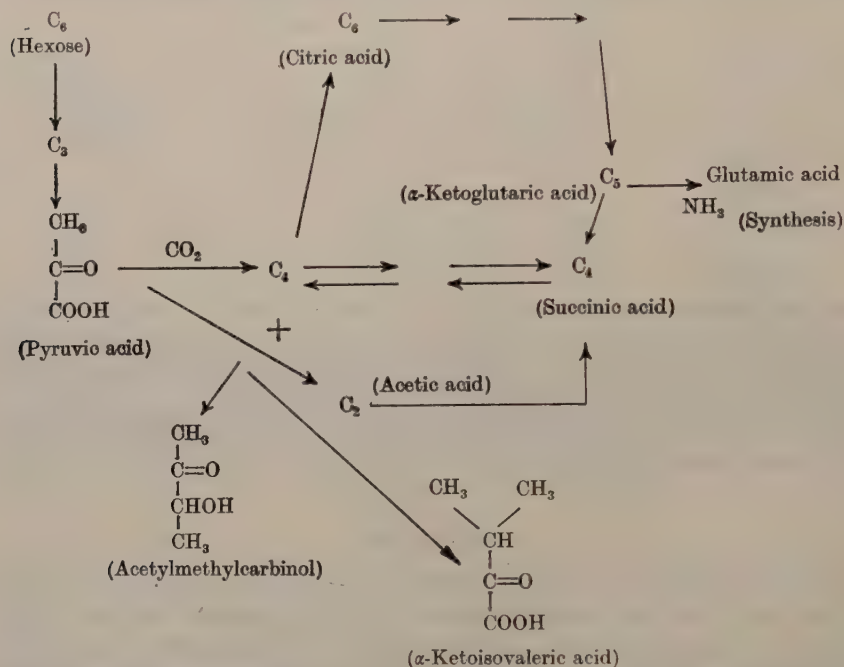


Fig. 9. Scheme of metabolism in the *suc* and *at suc* mutants of *Neurospora crassa*.

acid cycle (Peters, 1952), inhibits acetate oxidation by both wild-type and *suc* strains, and this inhibition is accompanied by an accumulation of citrate indicating that citrate is an intermediate in acetate oxidation by the *suc* mutants of *Neurospora*. Other mutants (of *Escherichia coli*) requiring dicarboxylic acids for growth and not responding to acetate have been described (Gilvarg & Davis, 1954), but there is presumably no use made of the tricarboxylic acid cycle in these strains since they are unable to oxidize acetate and lack condensing enzyme. Non-utilization of acetate for growth by strains requiring dicarboxylic acids, however, does not preclude use of the tricarboxylic acid cycle as a mechanism of acetate oxidation.

Pyruvic acid, AMC and C_2 condensation products which do not require complete oxidation accumulate in cultures of the *suc* mutants (necessarily grown in the presence of nitrogen salts) because of the deficiency in the dicarboxylic acid catalyst required for C_2 fragment oxidation. The same

accumulation products are produced in the *suc* mutants, where there is no immediate block to pyruvate oxidation as in the *ac* mutants (Strauss & Pierog, 1954), where there is a block in the oxidation of pyruvate: another illustration of the danger of using accumulations as evidence of genetic block (Adelberg, 1953). The relationship between succinate, a growth requirement, and AMC, a by-product of a true intermediate, is another case in which an end product controls the accumulation of a precursor (Strauss, 1955*a, b*; Adelberg & Umbarger, 1953). In this case, however, the 'end product' (succinate) is a catalyst for the metabolism of the intermediate.

At present it is not possible for the author to give an adequate explanation for the dicarboxylic acid deficiency of the *suc* mutants or to locate an absolute 'genetic block' in these strains. The tricarboxylic acid cycle operating in a complete cycle regenerates 1 mole of dicarboxylic acid for every mole supplied. Therefore, some other mechanism must be present to insure a net increase in the amount of dicarboxylic acids in an organism using the cycle for energy liberation and for the production of intermediates (Abelson & Vogel, 1955). At least two possible alternate mechanisms exist for the synthesis of C_4 acids; carbon dioxide fixation in a $C_3 + C_1$ type of condensation (Utter & Wood, 1951), and a $C_2 + C_2$ condensation by the Wieland-Thunberg reaction (Seaman & Naschke, 1955). However, the *suc* mutants are able to fix carbon dioxide, and the amount of carbon dioxide fixed in the acid-soluble fraction is increased on the addition of ammonium salts. The deficiency in the *suc* mutants is apparently in the incorporation of the fixation products into the non-soluble fraction (protein?), but this is what might be expected in any strain unable to grow.

A deficiency in the Wieland-Thunberg condensation might logically be expected in the *at* strain because of the lack of response of the *at suc* strain to acetate. However, Seaman (personal communication) has shown that both wild-type and mutant (*at suc*) preparations show comparable activities of the reversible succinate-cleaving enzyme.

Investigations in biochemical genetics seem to uncover two types of 'biochemical mutants'; one type showing a fairly simple relationship between gene, enzyme and growth requirement (Fincham, 1954; Davis, 1955), the other requiring a complex interpretation in terms of inhibitors and the balance of reactions (Mitchell & Mitchell, 1952). The advantage of studies of this latter type is that they show, somewhat more clearly than is possible by studies with the wild-type, the interactions between multi-enzyme systems (Dixon, 1949) which may be the basis for the intracellular regulation of metabolism (Strauss, 1955*b*). An effect that is present in 'wild-type' preparations, i.e. the ketogenic effect of ammonium (Recknagel & Potter, 1951) may be magnified in a mutant until a growth requirement occurs. Study of such mutants permits a more ready identification of the interactions actually regulating metabolism in normal organisms.

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A Proposed Revision of the Genus *Pullularia*

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SUMMARY: Morphological and physiological studies were made of 12 strains of the genus *Pullularia*. Although inability to ferment carbohydrates has been reported characteristic of this genus, 10 of the strains produced acid from one or more carbohydrates. It is proposed that the genus be emended to include the new species *P. fermentans*.

Lymph nodes in Hodgkin's disease have been the source of a variety of micro-organisms, including members of the genera *Bacillus*, *Brucella*, *Candida*, *Clostridium*, *Corynebacterium*, *Escherichia*, *Staphylococcus* and *Torula* (Hoster, Dratman, Craver & Rolnick, 1948; Haythorn, Robinson & Johnson, 1932). An organism recently isolated in our laboratory from a case of Hodgkin's granuloma appeared morphologically to be a *Pullularia* sp. However, this isolate produced acid from a number of carbohydrates; whereas the genus *Pullularia* has been described as not fermenting carbohydrates (Berkhout, 1923; Dodge, 1935). From culture collections there were obtained 11 additional strains designated as *Pullularia*. Of these, all but 2 fermented one or more carbohydrates. On the basis of morphological and physiological studies of these fungi to be reported in this paper, it is suggested that the genus *Pullularia* be emended to include fermenting forms as members of the proposed new species *P. fermentans*.

METHODS

The organism isolated in our laboratory was obtained from a mid-jugular lymph node of an 11-year-old boy with Hodgkin's granuloma. Paraffin sections stained with Gridley's fungus stain (Gridley, 1953) showed occasional conidia. A portion of the node was ground with sterile sand in a mortar, and the following media were inoculated: Sabouraud glucose agar (Difco), Lowenstein-Jensen medium (Difco), fluid thioglycollate medium (BBL), brain heart infusion broth (Difco) and blood agar. Organisms isolated were a Gram-negative rod, a staphylococcus and a black yeast-like fungus identified on morphological considerations as a *Pullularia* sp. However, the organism produced acid from a number of carbohydrates; whereas the genus *Pullularia* has been described as devoid of fermentative powers (Berkhout, 1923; Dodge, 1935). Therefore, a detailed morphological and physiological study was made of 11 strains of *Pullularia* obtained from culture collections. Two strains were secured from the American Type Culture Collection, Washington, and the other 9 from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. The organisms are listed with their sources in Table 1.

Table 1. Summary of biochemical and physiological reactions of 12 *Pullularia* strains

Source	Designation as received	Proposed designation	Fermentation of carbohydrates										Pigment	
CvS*	<i>P. pullulans</i> (de Bary) Berkhout strain Boedijn	<i>P. pullulans</i>	Glucose	+	+	+	+	+	+	+	+	+	Nutrient agar	+
CvS	<i>P. werneckii</i> (Horta) de Vries strain da Fonseca	<i>P. werneckii</i>	Glucose	+	+	+	+	+	+	+	+	+	Nutrient agar	+
Lymph node from Hodgkin's granuloma	Isolate	<i>P. fermentans</i> var. <i>fermentans</i>	Glucose	+	+	+	+	+	+	+	+	+	Nutrient agar	+
CvS	<i>P. pullulans</i> (de Bary) Berk. strain Margadent	<i>P. fermentans</i> var. <i>fermentans</i>	Glucose	+	+	+	+	+	+	+	+	+	Nutrient agar	+
CvS	<i>P. pullulans</i> (de Bary) Berk. var. <i>fusca</i> (Browne) Berk. strain Church	<i>P. fermentans</i> var. <i>fusca</i>	Glucose	+	+	+	+	+	+	+	+	+	Nutrient agar	+
ATCC†	<i>P. pullulans</i> (de Bary) Berk. 9348	<i>P. fermentans</i> var. <i>fusca</i>	Glucose	+	+	+	+	+	+	+	+	+	Nutrient agar	+
ATCC	<i>P. pullulans</i> (de Bary) Berk. 9349	<i>P. fermentans</i> var. <i>melinii</i>	Glucose	+	+	+	+	+	+	+	+	+	Nutrient agar	+
CvS	<i>P. pullulans</i> (de Bary) Berk. strain Melin	<i>P. fermentans</i> var. <i>melinii</i>	Glucose	+	+	+	+	+	+	+	+	+	Nutrient agar	+
CvS	<i>P. werneckii</i> (Horta) de Vries strain Leão	<i>P. fermentans</i> var. <i>leaoi</i>	Glucose	+	+	+	+	+	+	+	+	+	Nutrient agar	+
CvS	<i>P. pullulans</i> (de Bary) Berk. strain Schoen	<i>P. fermentans</i> var. <i>schoenii</i>	Glucose	+	+	+	+	+	+	+	+	+	Nutrient agar	+
CvS	<i>P. pullulans</i> (de Bary) Berk. strain Benedek	<i>P. fermentans</i> var. <i>benedekii</i>	Glucose	+	+	+	+	+	+	+	+	+	Nutrient agar	+
CvS	<i>P. pullulans</i> (de Bary) Berk. strain Castellani	<i>P. fermentans</i> var. <i>castellani</i>	Glucose	+	+	+	+	+	+	+	+	+	Nutrient agar	+
			Mannose	+	+	+	+	+	+	+	+	+		
			Fructose	+	+	+	+	+	+	+	+	+		
			Maltose	+	+	+	+	+	+	+	+	+		
			Sucrose	+	+	+	+	+	+	+	+	+		
			Raffinose	+	+	+	+	+	+	+	+	+		
			Xylose	+	+	+	+	+	+	+	+	+		
			Galactose	+	+	+	+	+	+	+	+	+		
			Rhamnose	+	+	+	+	+	+	+	+	+		
			Arabinose	+	+	+	+	+	+	+	+	+		
			Lactose	+	+	+	+	+	+	+	+	+		
			Growth at 37°	+	+	+	+	+	+	+	+	+		
			Anaerobic	+	+	+	+	+	+	+	+	+		

* Centraalbureau voor Schimmelcultures, Baarn (Netherlands).

† American Type Culture Collection, Washington, D.C.

Cultures were made on Littman oxgall agar (Difco), nutrient agar (Difco), Sabouraud glucose agar (Difco) and corn meal agar (Difco). These media were incubated at 25° and 37°, both aerobically and in an atmosphere of natural gas. Microscopic studies included examination of growth *in situ* on solid media, of temporary preparations consisting of growth from solid media suspended in water, and of slide cultures made from corn meal or Sabouraud agar. Tests for production of acid from carbohydrate were carried out in 10 ml. of phenol red broth base (Difco) to which a filtered solution of sugar had been added to a final concentration of 4%. After incubation for 4 weeks at a temperature of approximately 25°, the pH values of all tubes were determined with a Beckman Model G pH meter. Tubes with a pH value of 6·7 or lower were then titrated to pH 7·3 with 0·05 N-NaOH. A carbohydrate was considered to be fermented only when the final pH value was 6·6 or less, and 0·4 ml. or more of NaOH was required to adjust the pH to 7·3.

RESULTS AND DISCUSSION

On the basis of morphological considerations, all of the 12 cultures studied were considered as members of the genus *Pullularia*. Morphology of the strain isolated in our laboratory is illustrated in Pl. 1, and is representative of the group.

Of the 12 cultures which morphologically were essentially identical, 10 (including the isolated strain) produced acid from one or more carbohydrates, and therefore did not conform to published descriptions of the genus (Berkhout, 1923; Ciferri & Ashford, 1929; Dodge, 1935). Considerable variation was noted in the fermentation patterns of the various organisms. Variation was also noted in ability to grow at 37°, and in the production of pigment on nutrient agar or under anaerobic conditions (atmosphere of natural gas). The principal biochemical and physiological findings are summarized in Table 1.

To the authors' knowledge only two species are at present recognized in the genus *Pullularia*: *P. pullulans* (de Bary) Berkhout (Berkhout, 1923; Ciferri & Ashford, 1929; Dodge, 1935) and *P. werneckii* (Horta) de Vries (Horta, 1921; de Vries, 1952). On the basis of the physiological characteristics reported in this paper, it is proposed to emend the genus to include fermenting forms in a new species, *P. fermentans*, with 7 varieties (Table 1). The organism isolated in the present investigation is designated as the type for the species. Six of the 7 varieties are new, and the names of these were derived from the name of the investigator listed by the Centraalbureau voor Schimmelcultures as having furnished the strain. The seventh variety, *P. fermentans* var. *fusca*, represents a new combination, since the designation *P. pullulans* var. *fusca* (Browne) was used earlier by Berkhout (1923). The two strains from the American Type Culture Collection appear to belong to this variety.

The proposed revision of the genus *Pullularia* is admittedly based almost entirely on considerations of a physiological rather than a morphological nature. In this connexion it is of interest to note the recent emphasis upon physiological criteria in speciation of genera such as *Streptomyces* (Hesseltine,

Benedict & Pridham, 1954) and *Candida* (Lodder & Kreger-van Rij, 1952). Furthermore, it is recognized that strains of a single species such as *Penicillium chrysogenum* may exhibit pronounced morphological variation (Stauffer & Backus, 1954).

CLASSIFICATION

Pullularia Berkhout emend.

Pullularia Berkhout (1923)

= *Hormonema* Lagerberg, Lundberg & Melin (1927)

Type species *Pullularia pullulans* (de Bary) Berkhout (1923)

Colonies usually black, at first yeast-like but later velvety or woolly, margins often lighter than centres. Hyphae dark on most media. Mycelium at edges of colonies usually formed by lateral budding. Older portions of mycelium fragment into dark thick-walled arthrospores which 'sprout' to form blastospores. Thick-walled chlamydospores may also arise along the mycelium and are particularly numerous in old cultures. Uniseptate or cladosporium forms usually present. Pigment production varies with medium and oxygen tension. Most strains grow at 37°. Acid production from carbohydrates variable.

Key to *Pullularia*

- I. No acid from glucose.
 - A. Growth predominantly yeast-like, pigment on nutrient agar. *P. pullulans*
 - B. Growth predominantly mould-like, no pigment on nutrient agar. *P. werneckii*
- II. Acid from glucose.
 - A. No acid from mannose. *P. fermentans* var. *castellanii*
 - B. Acid from mannose.
 - 1. No acid from sucrose. *P. fermentans* var. *benedekii*
 - 2. Acid from sucrose.
 - a. No acid from fructose or maltose. *P. fermentans* var. *leaoi*
 - b. Acid from fructose and maltose.
 - (1) No acid from xylose.
 - (a) No acid from raffinose, growth at 37°. *P. fermentans* var. *schoenii*
 - (b) Acid from raffinose, no growth at 37°. *P. fermentans* var. *fermentans*
 - (2) Acid from xylose.
 - (a) No acid from rhamnose, no pigment on nutrient agar. *P. fermentans* var. *fusca*
 - (b) Acid from rhamnose, pigment on nutrient agar. *P. fermentans* var. *melinii*

Descriptions of species

Pullularia pullulans (de Bary) Berkhout (1923)

≡ *Dematium pullulans* de Bary (in Loew, 1868)

Blastospores 3–4 × 7–11 μ., chlamydospores 14 μ., arthrospores 3–4 × 7–10 μ., cladosporium forms 3–5 × 12–14 μ. Small black colonies with greenish black surface and aerial mycelium on Sabouraud, corn meal, nutrient and Littman agar. Pigment produced only anaerobically. Growth at 37°. Pellicle in broth with slight sediment. Carbohydrates not fermented.

Pullularia werneckii (Horta) de Vries (1952)= *Caraté noir* Montoya y Florez (1898)= *Montoyella nigra* Castellani & Chalmers (1913)= *Cladosporium wernecki* Horta (1921)= *Dematium wernecki* (Horta) Dodge (1935)

Blastospores $3 \times 7-10 \mu$., chlamydospores $6-7 \mu$., arthrospores $3-4 \times 7-11 \mu$., cladosporium forms $3-4 \times 10 \mu$. Black colonies with dark and light aerial mycelium on Sabouraud, corn meal and Littman agar. Mould type growth. Pigment produced aerobically or anaerobically, but not on nutrient agar. Growth at 37° . Carbohydrates not fermented.

Pullularia fermentans sp.nov. (Pl. 1)

Blastospores $3-4 \times 7-10 \mu$., chlamydospores $13-15 \mu$., arthrospores $5-7 \times 10-14 \mu$., cladosporium format $9 \times 17 \mu$. Coloniae nigrae cum mycelio albo in superficie in agaro Sabouraudi et agaro farinae frumenti. Pigmentum solum cum oxygenio effectum, variabile in agaro Littmani et absens in agaro nutriente. Conformatio mycelii aerii variabilis. Non crescit cum 37° . Glucosum, mannosum, fructosum, maltosum, sucrosum et raffinatum fermentat.

Blastospores $3-4 \times 7-10 \mu$., chlamydospores $13-15 \mu$., arthrospores $5-7 \times 10-14 \mu$., cladosporium forms $9 \times 17 \mu$. Black colonies with white surface mycelium on Sabouraud and corn meal agar. Pigment produced only aerobically, variable on Littman agar and absent on nutrient agar. Production of aerial mycelium variable. No growth at 37° . Ferments glucose, mannose, fructose, maltose, sucrose and raffinose.

This type strain was isolated by the authors in 1954 from a mid-jugular lymph node of an 11-year-old boy with Hodgkin's granuloma.

Pullularia fermentans Wynne et Gott var. *fermentans*

Characters as for *P. fermentans*

Pullularia fermentans Wynne et Gott var. *fusca* (Browne) comb.nov.= *Monilia fusca* Browne (1918)= *Pullularia pullulans* (de Bary) Berkhout var. *fusca* (Browne) Berkhout (1923)

Blastospores $3-4 \times 11-13 \mu$., chlamydospores $13-16 \mu$., arthrospores $3-6 \times 8-12 \mu$., cladosporium format $4-6 \times 14 \mu$. Coloniae nigrae mucosae cum mycelio nigro in superficie in agaro Sabouraudi, farina frumenti et in agaro Littmani. Pigmentum variabile in conditionibus sine oxygenio, absens in agaro nutriente. Mycelium aerium non observatum est. Crescit cum 37° . Circuli pellicularum in decocto. Glucosum, mannosum, fructosum, maltosum, sucrosum, raffinatum et xylosum fermentat.

Blastospores $3-4 \times 11-13 \mu$., chlamydospores $13-16 \mu$., arthrospores $3-6 \times 8-12 \mu$., cladosporium forms $4-6 \times 14 \mu$. Black mucoid colonies with black surface mycelium on Sabouraud, corn meal and Littman agar. Pigment variable under anaerobic conditions, absent on nutrient agar. Aerial mycelium not observed. Growth at 37° . Rings of pellicles in broth. Ferments glucose, mannose, fructose, maltose, sucrose, raffinose and xylose.

Type strain obtained from Centraalbureau voor Schimmelcultures as *Pullularia pullulans* (de Bary) Berkhout var. *fusca* (Browne) Berkhout (1923).

***Pullularia fermentans* Wynne et Gott var. *melinii* var. nov.**

Blastosporae $3-5 \times 7-13 \mu$., chlamydosporae $13-16 \mu$., arthrospora $3 \times 14 \mu$., cladosporium format $5-8 \times 10-17 \mu$. Coloniae nigrae mucosae cum mycelio nigro in agaro Sabouraudi, farina frumenti et agaro nutriente. Nullum pigmentum sine oxygenio. Nullum mycelium aerium. Crescit cum 37° . Circuli pellicularum in decocto. Glucosum, mannosum, fructosum, maltosum, sucrosum, xylosum, galactosum et rhamnosum fermentat.

Blastospores $3-5 \times 7-13 \mu$., chlamydospores $13-16 \mu$., arthrospores $3 \times 14 \mu$., cladosporium forms $5-8 \times 10-17 \mu$. Black mucoid colonies with black mycelium on Sabouraud, corn meal and nutrient agar. No pigment anaerobically. No aerial mycelium. Growth at 37° . Rings of pellicles in broth. Ferments glucose, mannose, fructose, maltose, sucrose, xylose, galactose and rhamnose.

Obtained from Centraalbureau voor Schimmelcultures as *Pullularia pullulans* (de Bary) Berkhout strain Melin. Received by the Centraalbureau in 1929 as *Hormonema dematioides*.

***Pullularia fermentans* Wynne et Gott var. *leaoi* var. nov.**

Blastosporae $3-4 \times 7-10 \mu$., chlamydosporae 12μ ., arthrospora $4 \times 4-7 \mu$., cladosporium format $3 \times 11-14 \mu$. Coloniae subvirides nigrae cum mycelio aereo in agaro Sabouraudi et agaro farinae frumenti, coloniae nigrae in agaro Littmani. Pigmentum cum aut sine oxygenio, absens in agaro nutriente. Non crescit cum 37° . Crescit in decocto solum qualis pellicula. Glucosum, mannosum, sucrosum et raffinose fermentat.

Blastospores $3-4 \times 7-10 \mu$., chlamydospores 12μ ., arthrospores $4 \times 4-7 \mu$., cladosporium forms $3 \times 11-14 \mu$. Greenish black colonies with aerial mycelium on Sabouraud and corn meal agar, black colonies on Littman agar. Pigment aerobically or anaerobically, absent on nutrient agar. No growth at 37° . Growth in broth as pellicle only. Ferments glucose, mannose, sucrose and raffinose.

Obtained from Centraalbureau voor Schimmelcultures as *Pullularia werneckii* (Horta) de Vries strain Leão. Received by the Centraalbureau in 1948 from Leão and Cury, who isolated it from a case of tinea nigra (keratomycosis nigricans palmaris).

***Pullularia fermentans* Wynne et Gott var. *schoenii* var. nov.**

Blastosporae $3-4 \times 7-12 \mu$., chlamydosporae $13-14 \mu$., arthrospora $8-11 \times 10-13 \mu$., cladosporium format $7 \times 14-17 \mu$. Coloniae nigrae viscosae cum mycelio nigro in superficie et mycelio viridi aereo in agaro Sabouraudi et agaro farinae frumenti, coloniae nigrae scabrae cum mycelio albo aereo in agaro Littmani. Pigmentum solum cum oxygenio effectum, absens in agaro nutriente. Crescit cum 37° . Circuli pellicularum in decocto. Glucosum, mannosum, fructosum, maltosum et sucrosum fermentat.

Blastospores $3-4 \times 7-12 \mu$., chlamydospores $13-14 \mu$., arthrospores $8-11 \times 10-13 \mu$., cladosporium forms $7 \times 14-17 \mu$. Viscous black colonies with black surface mycelium and green aerial mycelium on Sabouraud and corn meal agar, rough black colonies with white aerial mycelium on Littman agar. Pigment

produced only aerobically, absent on nutrient agar. Growth at 37°. Rings of pellicles in broth. Ferments glucose, mannose, fructose, maltose and sucrose.

Obtained from Centraalbureau voor Schimmelcultures as *Pullularia pullulans* (de Bary) Berkhout strain Schoen. Received by the Centraalbureau in 1937 as *Torula schoenii* Roukhelman.

***Pullularia fermentans* Wynne et Gott var. *benedekii* var. nov.**

Blastosporae $3-5 \times 7 \mu$., chlamydosporae 12μ ., arthrospora $3-5 \times 5-7 \mu$., cladosporium format $3 \times 10 \mu$. Coloniae nigrae cum mycelio aereo cinereo aut mycelio nigro in superficie in agar Sabouraudi, farina frumenti, nutrimento et agar Littmani, tantum sine oxygenio quantum cum oxygenio. Crescit cum 37°. Parum formata pellicula et cristae incrementi per totam substantiam liquidam. Glucosum et mannosum fermentat.

Blastospores $3-5 \times 7 \mu$., chlamydo-spores 12μ ., arthrospores $3-5 \times 5-7 \mu$., cladosporium forms $3 \times 10 \mu$. Black colonies with grey aerial mycelium or black surface mycelium on Sabouraud, corn meal, nutrient and Littman agar, both anaerobically and aerobically. Growth at 37°. Poorly formed pellicle and tufts of growth throughout liquid medium. Ferments glucose and mannose.

Obtained from Centraalbureau voor Schimmelcultures as *Pullularia pullulans* (de Bary) Berkhout strain Benedek. Received by the Centraalbureau in 1933 as *Torula lecanii corni*, isolated from *Lecanium corni*.

***Pullularia fermentans* Wynne et Gott var. *castellani* var. nov.**

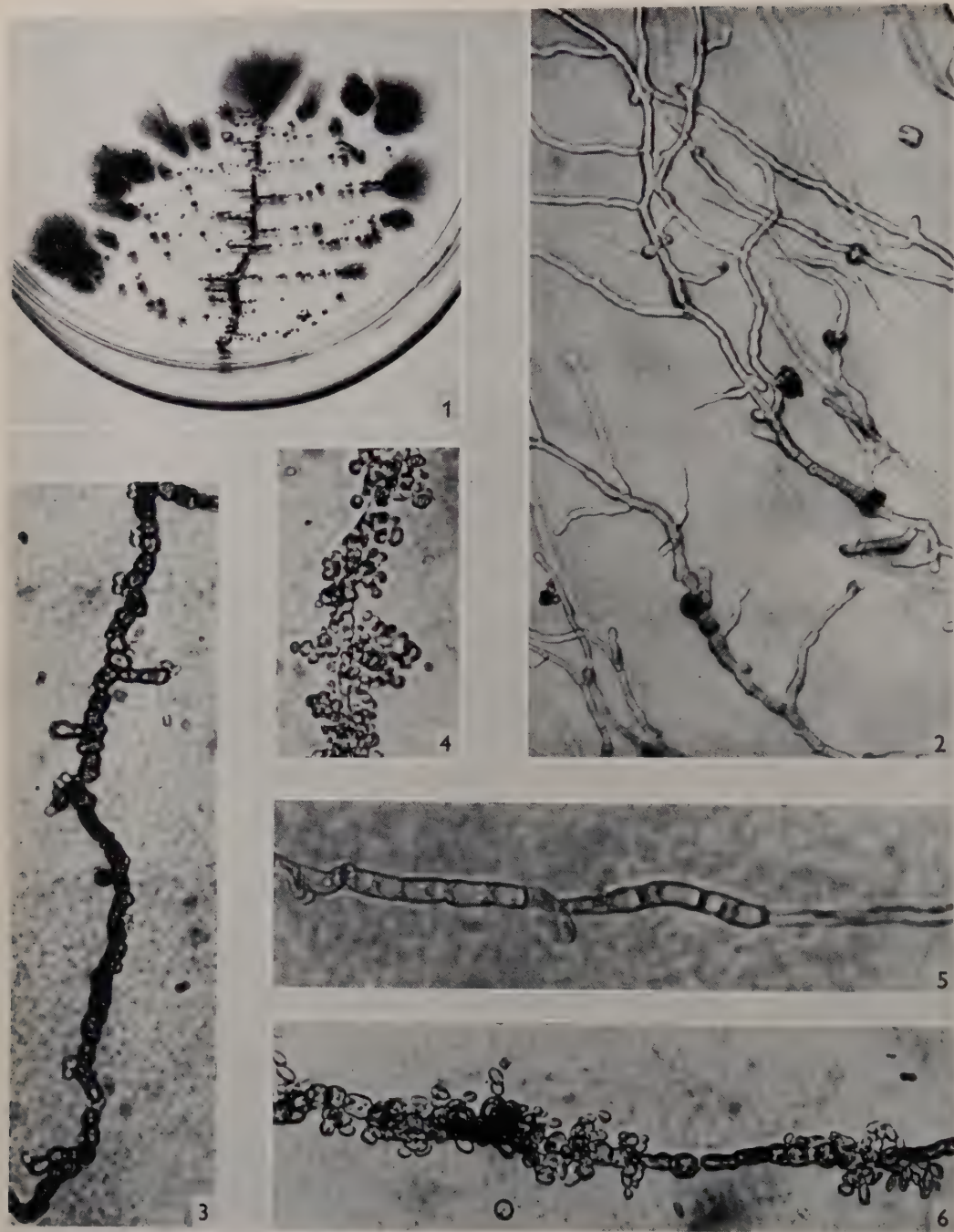
Blastosporae $3-4 \times 7-10 \mu$., chlamydosporae $12-13 \mu$., arthrospora $3 \times 10-13 \mu$., cladosporium format $3 \times 11 \mu$. Coloniae nigrae cum mycelio viridi aereo et in superficie agar Sabouraudi, farina frumenti, nutrimento et agar Littmani. Nullum pigmentum sine oxygenio. Non crescit cum 37°. Nulla pellicula in decocto; coloniae cristatae per totam substantiam. Glucosum solum fermentat.

Blastospores $3-4 \times 7-10 \mu$., chlamydosporae $12-13 \mu$., arthrospores $3 \times 10-13 \mu$., cladosporium forms $3 \times 11 \mu$. Black colonies with green aerial and surface mycelium on Sabouraud, corn meal, nutrient and Littman agar. No pigment anaerobically. No growth at 37°. No pellicle in broth; tufted colonies throughout medium. Ferments glucose only.

Obtained from Centraalbureau voor Schimmelcultures, who received it in 1935 as *Cryptococcus metaniger*. Isolated by Castellani (1927) from a case of trichomycosis nigra.

Cultures of the type strain of *Pullularia fermentans* and the type variety and the type strain of each of its other 6 varieties have been dispatched to the American Type Culture Collection, The Commonwealth Mycological Institute Collection of Fungus Cultures, and the Centraalbureau voor Schimmelcultures.

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E. S. WYNNE AND C. L. GOTT—PROPOSED REVISION OF PULLULARIA. PLATE 1

(Facing p. 519)

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EXPLANATION OF PLATE 1

Pullularia fermentans sp.n. isolated from cervical lymph node of a case of Hodgkin's granuloma. All photomicrographs at approximately $\times 800$.

- Fig. 1. Colonies on corn meal agar after 12 days at 25°.
- Fig. 2. Young mycelium with early blastospores from edge of colony on corn meal agar. Portions of the mycelium and some of the blastospores are dark because of beginning pigment production.
- Fig. 3. Older mycelium completely fragmented into dark arthrospores, some of which exhibit characteristic 'sprouting'.
- Fig. 4. Masses of blastospores resulting from sprouting of arthrospores.
- Fig. 5. Formation of double-walled chlamydospores.
- Fig. 6. Arthrospores, blastospores and chlamydospores in a single hyphal strand.

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Some Observations on the Antigenic Structure of Trichloroacetic Acid Extracts in *Escherichia* and the Paracolon Group

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SUMMARY: All the somatic antigens of 'smooth' type of some *Escherichia* and paracolon strains studied are not extracted by trichloroacetic acid (TCA), though they may be by boiling or by mechanical disruption of the cell.

The term 'paracolon' is passing out of common use and is applied here descriptively rather than definitively. Some strains were encountered which were members of the family Enterobacteriaceae and resembled coliform organisms in their morphological and cultural characteristics; they were late, or non-lactose-fermenters, and did not belong to *Escherichia*, Providence, Bethesda-Ballerup or other similar groups. Interest in the group was stimulated by an unusual case which has been reported elsewhere (Macpherson & Exner-Baumann, 1950). At this time, investigators were pessimistic about the possibility of classifying the paracolon group by conventional serological methods (e.g. Stuart, Galton & McGann, 1948; Mushin, 1949). The use of trichloroacetic acid (TCA) extracts as suggested by Boivin, Mesrobianu, Magheru & Magheru (1935) was considered. The success which had attended their employment in *Pseudomonas* (van den Ende, 1952) was felt to be encouraging and the technique was accordingly adopted.

Some success was obtained and the serological classification of biochemically homogeneous groups was possible. This classification was, of course, based on the somatic antigenic structure of the strains and could be confirmed and amplified by the use of 'O' and 'H' antisera (Macpherson, 1954). Anomalies were encountered, however, and it appeared that they were due either to the failure of the TCA to extract certain somatic antigens, or to the presence of antigens of 'envelope' type.

To confirm either of these possibilities, it was necessary to employ strains which shared labelled somatic or envelope antigens. By preparing antisera against such strains and then studying the cross-reactions obtained with TCA extracts, it should be evident whether some, all or no such antigens were being extracted. Since suitable paracolon strains were not available, *Escherichia* strains were obtained from Dr F. Kauffmann of Copenhagen.

It has been emphasized, almost since antigenic extracts were first studied, that they are not chemically homogeneous, and the occurrence of degradation

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from full antigenicity is also well documented. However, such apparently capricious differentiation between presumably similar antigens, some being extracted and others not, does not appear to have been described before.

METHODS

The main antigens of the *Escherichia* strains are shown in Table 1. Cultures, after testing for purity, were dried by the method of Stamp (1947) and a fresh pellet used for each experiment. TCA extracts were prepared by the method of Boivin, Mesrobianu & Mesrobianu (1933), but no attempt was made to purify the extracts or to concentrate them.

Table 1. *Short antigenic formulae of Escherichia strains employed (Kauffmann's notation)*

Strain	Antigens		
	O	K	H
O 1	1	L 1	7
O 2	2	L 1	4
H 6	2	L 1	6
K 7	7	L 7	4
K 25	8	B 25	9
K 48	8	A 48	9

Antisera were prepared in rabbits. For paracolon, and *Escherichias* 'H' sera, overnight broth cultures were used, formalin having been added to a final concentration of 1 % (v/v). Saline suspensions of overnight agar cultures were used for the preparation of *Escherichias* 'OK' sera. Intravenous injections of 0.25, 0.5, 1.0, 1.5 and 2.0 ml. were given at 5-day intervals, and animals bled from the ear vein 7 days after the last injection. Strain K 48, which has an envelope antigen of 'A' type, was plated out and both non-capsulated and capsulated forms selected. Injected into separate rabbits, these gave rise to almost pure somatic and envelope antisera, labelled 'A—' and 'A+' respectively. The titre of 'O' and 'H' antibodies was in the region of 1/2000 but, as is usual, the titre of 'K' antibodies was much lower.

Antisera were absorbed with the centrifuged deposit from an overnight broth culture. If living cultures were used to absorb an 'OK' serum, pure 'O' sera resulted (e.g. K 25 'O' serum). If the culture was heated at 100° for 2½ hr. before absorption, or if a non-capsulated form was used, pure 'K' sera could be obtained (e.g. K 25 'K'). The proportion of serum to organisms was very important. The deposit of organisms was taken up in 1.5 ml. of serum which had been diluted 1/5 with a 50 % (v/v) glycerol-saline mixture. After incubation at 37° for 2 hr. and overnight at 2° the mixture was centrifuged and the supernatant fluid removed. Varying the relative amounts within fairly narrow limits gave rise to frequent anomalous results. Glycerol-saline had to be used for the dilutions since use of a fluid of lower viscosity made it impossible to layer the TCA extracts on top of the serum for the ring precipitation tests which were used throughout.

Agglutination tests were by the tube method, equal parts of serum and suspensions being used. O antigens were incubated at 37° for 18 hr., L and B antigens for 2 hr. at 37° and 20 hr. at room temperature, A antigens for 20 hr. at 50° and H antigens for 2 hr. at 50° and then overnight at room temperature. All tests were read macroscopically.

Mechanical disintegration of cultures was by the technique of Salton & Horne (1951), using glass ballotini in a Mickle disintegrator followed by centrifugation at 10,000 r.p.m. for 15 min. to sediment the bacterial debris.

RESULTS

TCA extracts of the strains shown in Table 1 were tested against the antisera prepared from these strains, with the results shown in Table 2. It will be seen that cross-reaction occurs between the extracts from strains O2 and H6, which share O antigen 2, but that there is no cross-reaction between strains K 25 and K 48, which share O antigen 8. There is no evidence that any envelope or flagellar antigen is present in the extracts.

Table 2. *Reactions between TCA extracts and antisera of strains in Table 1*

Antisera	TCA extracts of strains						
	O1	O2	H6	K7	K25	K48A+	K48A-
O1	++	-	-	-	-	-	-
O2	-	++	+	-	-	-	-
H6	-	+	++	-	-	-	-
K7	-	-	-	++	-	-	-
K25	-	-	-	-	++	-	-
K48A+	-	-	-	-	-	++	±
K48A-	-	-	-	-	-	++	++

++ = immediate, marked ring of precipitation; + = immediate, but only slight, ring of precipitation; ± = slight ring, taking more than 5 min. to develop; - = no ring of precipitation after 45 min.

Since these latter two strains should, theoretically, have cross-reacted, agglutination tests were put up, using both the ordinary K 25 'OK' serum and absorbed sera. 'OK' sera were absorbed both with living and with heated suspensions of their homologous strains to produce pure 'O' and 'K' sera. It is evident that there is marked cross-agglutination due to sharing of O antigen 8 (Table 3).

A further method of investigating this anomaly was suggested by findings in the earlier studies with paracolon strains. The polysaccharide of the TCA extracts had been found to be very resistant to heating at 100° or 120°, no detectable, or minimal fall in titre resulting. If, however, the cell suspension was heated before extracting with TCA, the extract either failed to precipitate, or reacted very feebly with homologous serum. The reason for this was not that the polysaccharide was being altered or destroyed within the body of the organism, but that it was being extracted by the heating process, regardless of the type of fluid in which the organisms were suspended. A number of experi-

ments designed to prove this point did so unequivocally, and a representative experiment is described below.

By serial subculture, with agitation, a heavily turbid culture late in the logarithmic phase was prepared, and was divided into four equal parts. The first part was extracted with TCA in the usual way. Part 2 was boiled for 1 hr. and centrifuged. The supernatant fluid was retained and the pellet extracted

Table 3. *Reactions between K 25 and K 48 antisera, and heated and unheated agglutinable suspensions*

Antisera	Suspensions			
	K 48A +	Heated K 48A +	Heated K 25	K 25
K 48 pure 'O'	—	++	++	±
K 48 pure 'K'	+	—	—	—
K 25 'OK' serum	—	++	++	+
K 25 pure 'O'	—	++	++	±
K 25 pure 'K'	—	—	—	+

Preparation and notation of absorbed sera—see 'Methods'. Precipitation reactions—as for Table 2. Agglutination reactions: ++ = agglutination at a dilution of 1/200 or higher; + = no agglutination above 1/20 dilution; ± = no agglutination above 1/2 dilution; — = no agglutination at any dilution.

with TCA. Part 3 was also boiled for 1 hr. and centrifuged. The supernatant fluid was discarded and the pellet was mechanically disintegrated. After further centrifugation, the supernatant fluid was retained and the pellet extracted with TCA. Part 4 was mechanically disintegrated (without prior boiling) and centrifuged. The supernatant fluid was retained and the pellet extracted with TCA. All these fluids were then tested against homologous serum, with the results shown in Table 4. It is evident that boiling removes practically all the

Table 4. *Effect of heat and/or mechanical disintegration prior to TCA extraction*

Type of fluid tested	Homologous serum
Normal TCA extract	++
SNF after boiling culture for 1 hr.	++
TCA extract after boiling culture	±
SNF after mechanical disintegration, following boiling	±
TCA extract after boiling and mechanical disintegration	—
SNF after mechanical disintegration only	++
TCA extract after mechanical disintegration only	—
SNF after standing at room temperature for 6 hr.	—

SNF = supernatant fluid pipetted off after centrifugation. Precipitation reactions—as for Table 2.

TCA-extractable and mechanically-extractable material, while mechanical disintegration removes all the TCA-extractable material. The presence, and survival, of precipitable material in the various fluids shows that in each case the process is one of extraction, and not of destruction.

One interesting finding was that the antibody-binding power of a control culture was not detectably different before and after boiling.

An obvious possibility, in view of the above findings, was that in preparation, by heating, of O-agglutinable suspensions in *Escherichia* strains possessing an envelope antigen of 'L' type (Kauffmann, 1951), the envelope antigen might be completely extracted into the fluid, rather than being destroyed. However, application of the above technique to *Escherichia* strains K 25 and K 48, using the absorbed sera prepared for the first experiment, showed that this was not the case (Table 5). This table also shows that O antigen 8 is extracted by heating and that it is then freely cross-precipitated by the heterologous serum. Titration of these 'heat-extracts' with heterologous and homologous serum gave a much lower titre in the former, indicating that O antigen 8 can form only a small part of the precipitating material.

Table 5. *Precipitation reactions between K 25 and K 48 absorbed sera, and supernatant fluids from heated cultures*

Antisera	Supernatant fluids from heated cultures		
	K 48A +	K 48A -	K 25
K 48, pure 'O'	++	++	++
K 48, pure 'K'	-	-	-
K 25, pure 'O'	++	++	++
K 25, pure 'K'	-	-	-

Absorbed sera—for preparation and notation, see under 'Methods'. Precipitation reactions—as for Table 2.

To summarize, it could be shown that in these two *Escherichia* strains, O antigen 8 was extractable by heating and mechanical disintegration but not by TCA, while the other somatic antigens were freely extractable by all three methods. Neither heat nor TCA extracted antigens of envelope type.

Confirmation was thus obtained of the hypothesis which had been advanced to explain the anomaly found in the earlier paracolon studies. The anomaly was that mirror absorption tests had shown the TCA extracts of a group of 6 strains to be identical. Agglutination tests showed that the first five members of the group were very similar to each other but shared only minor antigens with the sixth member, cross-agglutinating only at dilutions of 1/20 or less (Table 6).

Table 6. *Comparison of agglutination and precipitation reactions in a group of 6 paracolon strains, using absorbed and unabsorbed sera*

Antisera	Agglutinating suspensions		TCA extracts	
	1	Group	1	Group
1	1/2000	1/20	++	++
Group	-	1/2000	++	++
1-1	-	-	-	-
1-Group	1/2000	-	-	-
Group-Group	-	-	-	-
Group-1	-	1/2000	-	-

'Group' indicates any member of the subgroup of 5 strains—all reacted similarly. '1' indicates the sixth member of the group. Agglutination tests—as for Table 3. Precipitation tests—as for Table 2. '1-group' indicates that serum 1 has been absorbed by a suspension of one or other strain in the subgroup of 5 strains.

From this table it is evident that however minor an agglutininogen this antigen may be, it is the only one which is extracted in all six strains. This is proved by the ability of any strain to exhaust the precipitins (as distinct from the agglutinins) from any heterologous serum.

A similar anomaly found in another group of paracolon strains may be explained by the fact that the TCA extraction method was not sufficiently sensitive. The antigen was present in minute amounts, being just detectable by mechanical disintegration but not by heat-extraction.

DISCUSSION

The most important finding is that TCA may fail to extract one or more of the somatic antigens in an organism while extracting the rest quite satisfactorily; no explanation for this phenomenon can be offered. It constitutes a strong argument against the use of TCA extracts for serological classification, unless the results are controlled by other methods. It would not, however, invalidate the use of TCA extracts of proven antigenic structure for rapid screening of large numbers of sera.

In both the *Escherichia* and paracolon groups, marked discrepancies were found between the antigens in question, as judged by the precipitation and agglutination tests, and it is interesting to speculate how much of this can be explained by a superficial or deep situation in the wall of the organism. The most reasonable explanation of the agglutininogenic dominance of *Escherichia* O antigen 8, which is present in only small amount in the extracts, would seem to be that it is superficially situated.

The fact that heat-extraction of antigens produces no detectable diminution in antibody-binding capacity would suggest that comparatively little of the available antigen is normally concerned in this function. However, heat-extraction may offer an explanation for Kauffmann's observation that 'when working with a non-flagellated acapsular strain, a stronger O serum is obtained if the strain is killed with 0.5 % formalin and not by heating'.

The failure of TCA to extract the envelope antigens of these *Escherichia* strains is interesting, contrasting as it does with TCA's known ability to extract the Vi antigen of *Salmonella typhi* (Combiesco, Combiesco & Soru, 1937; Boivin & Mesrobian, 1938).

Since decisions on relationships between strains, and even groups, of bacteria are often influenced by the dominance of one or other antigen as an agglutininogen, it seems possible that this lack of correlation between the results in the precipitin and agglutinin tests may be of taxonomic significance.

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Cytological Effects of Ultraviolet Radiation and Azaserine on *Corynebacterium diphtheriae*

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SUMMARY: Ultraviolet irradiation and the radiomimetic antibiotic substance L-azaserine have similar effects in promoting the formation of radially enlarged cells and elongated filaments, and in the accumulation of metaphosphate in metachromatic granules, with a strain of *Corynebacterium diphtheriae*. Dosages effective for the formation of enlarged cells and filaments are lower than for stimulation of metaphosphate accumulation.

Attention has been directed to parallel effects on bacterial cells of ultraviolet radiation and of certain radiomimetic substances which possess carcinogenic or oncolytic action (Lwoff, 1953; Kellenberger, 1953). Similar parallel effects may now be cited for u.v. radiation and L-azaserine (*o*-diazoacetylserine), an antibiotic substance which has anti-neoplastic properties (Stock, Reilly, Buckley, Clarke & Rhodes, 1954). Azaserine is mutagenic (Demerec *et al.* 1954), capable of inducing filament formation in *Escherichia coli* (Maxwell & Nickel, 1954), and of inducing development of lytic phage in lysogenic *E. coli*, strain K-12 (Gots, Bird & Mudd, 1955). The present communication is concerned with the cytological effects of u.v. radiation and azaserine on *Corynebacterium diphtheriae*.

METHODS

A mitis strain, A 9255, of *Corynebacterium diphtheriae*, whose cytology has been studied in detail (Davis & Mudd, 1955) was used throughout. Organisms were initially grown for approximately 18 hr. in M. & E. (Morton & Engley, 1945) broth containing 5% horse blood serum, and at the time of treatment with u.v. radiation or azaserine, lacked metachromatic granules or were poorly metachromatic.

The phenomena to be described were observed following irradiation of organisms, either suspended in 10 ml. of various solutions in Petri dishes (saline, phosphate, sucrose, distilled water or Tris buffer (2-amino-2-hydroxy methyl-1.3 propandiol, obtained from Sigma Chemical Co., St Louis 13, Missouri)), dried down on agar plates, or initially lyophilized and the dry organisms spread on Petri dishes for irradiation prior to reconstitution in distilled water. Organisms were also exposed to various concentrations of azaserine diluted in Tris buffer, and were then washed and incubated. All organisms were incubated on blood agar after treatment and checked periodically for cytological changes, using Neisser's acidified methylene blue stain.

As a source of u.v. radiation a General Electric germicidal lamp (G 15 T 8) was used whose output is predominantly at 2537 Å. The lamp in most of the

experiments was 18.7 in. from the organisms. Under these conditions the radiation, as measured by a General Electric germicidal u.v. intensity meter 16522, was approximately 105 u.v. mW./sq.ft. (1130 ergs/sec./cm.²). In certain experiments the u.v. source was 1 in. from the organisms; the intensity of irradiation was then estimated as 2.9 u.v. W./sq.ft. (31,215 ergs/sec./cm.²).

Azaserine, kindly supplied by Parke, Davis and Co., was used in the concentrations indicated in Fig. 1 and in the text.

Cytological observations were made with the aid of redox indicators. Suitable concentrations in M. & E. broth were 0.1 % neotetrazolium chloride, 0.02 % blue tetrazolium chloride, 0.05 % potassium tellurite, and 0.002 % Janus green B. Also employed were Sudan black B-citric acid (Davis & Mudd, 1955), the Hale (1953) cell wall stain, and nuclear stains according to Feulgen and DeLamater (1951).

A modification of the May-Grünwald stain (Hartman & Payne, 1954) was further modified so as to permit differential staining of nuclei and metachromatic granules in *Corynebacterium diphtheriae*. The modified procedure is as follows:

- (1) Fix coverslip impression smears of organisms overnight in methanol previously chilled on dry ice; transfer for a few seconds to methanol at room temperature.

- (2) Stain smears 10 min. in 1 : 5 dilution in methanol of a saturated solution of methylene blue eosinate made up in methanol. (The saturated solution was aged for at least 2 weeks before use.) Rinse in distilled water.

- (3) Stain smears 10 min. in 1 : 10 Giemsa solution in distilled water. Rinse.

- (4) Hold smears 1 min. in 5 % citric acid to remove dense cytoplasmic staining.

- (5) Blot, rinse twice in xylol, and mount in Harleco synthetic resin.

RESULTS

Fig. 1 shows the effects of u.v. irradiation and azaserine upon viability and the formation of radially enlarged organisms and elongated filaments. Pl. 1 shows some of the cytological changes involved. Photographs of azaserine-treated organisms were not included because of similarity to those of irradiated organisms.

Enlarged organisms and filaments. With relatively short periods of irradiation (5–20 sec. at 18.7 in.) cells were apparently unchanged (Fig. 1, curve A). With longer exposure (40–120 sec.) organisms were observed to undergo elongation which at times was accompanied by enlargement to abnormal size (Pl. 1, fig. 2). Formation of enlarged organisms soon ceased altogether when irradiation times were extended beyond 2 min., and most organisms were unable to produce colonies (Pl. 1, fig. 3). Persistently enlarged organisms could be obtained by exposing organisms to repeated doses of u.v. with intermittent incubation on blood agar (Pl. 1, figs. 4, 5). However, when doses which were optimal for producing, such organisms were used repeatedly, the organisms lost their ability to segment in an orderly fashion and instead showed extensive fragmentation and disintegration.

Cytological observation of enlarged organisms coloured with either the tetrazoles or potassium tellurite, or stained with Sudan black B-citric acid showed that they contained more redox-active lipid-containing granules than did the normal organisms of smaller size (Pl. 1, figs. 6*a*, *b*). Enlarged organisms often lacked septa, although some possessed one or two (Pl. 1, fig. 6*a*). Nuclear stains showed that extensive cellular elongation and enlargement was accompanied by dispersion of nuclear material throughout the organisms, either in discrete areas or in long continuous masses which in time might become segmented. The modified May-Grünwald stain was able to distinguish red-staining nuclear material from purple-staining metachromatic granules (Pl. 1, figs. 5, 7-10).

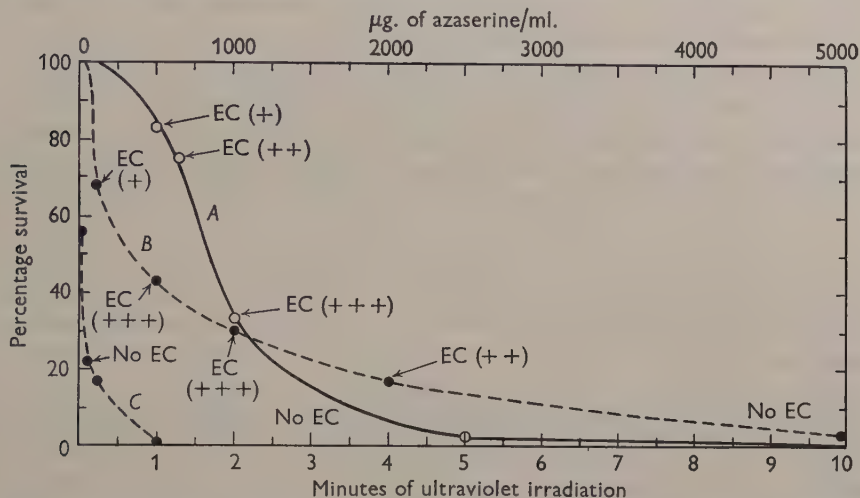


Fig. 1. *Corynebacterium diphtheriae*, strain mitis A9255, originally grown 12 hr. in blood broth, shows effect of u.v. irradiation and exposure to azaserine upon cell viability, as determined by plate counts, and formation of enlarged cells. Curve *A*=organisms irradiated with germicidal lamp at 18.7 in., then incubated upon blood agar. Curve *B*=organisms suspended in azaserine solution for 1 hr., then washed and incubated on blood agar. Curve *C*=as *B* but organisms incubated in azaserine solution for 5 hr. EC=enlarged cell formation as indicated by enlargement and filament formation. +, ++, +++=degree of such formation.

Treatment of organisms with azaserine for 1 hr. (Fig. 1, curve *B*) in concentration of 50 $\mu\text{g./ml.}$ did not noticeably affect them. Concentrations of 500-1000 $\mu\text{g./ml.}$ promoted enlargement and decreased survival rate. Formation of enlarged organisms was less evident at 2000 $\mu\text{g./ml.}$ and absent at 5000 $\mu\text{g./ml.}$ When organisms were exposed to azaserine for 5 hr. (Fig. 1, curve *C*) there was a rapid loss of viability, 500 $\mu\text{g./ml.}$ killing over 99%, and the ability to produce enlarged organisms was lost.

Metachromatic granules. In organisms irradiated for as long as 100 sec. or more, metachromatic granules were observed to form more rapidly and attain a greater ultimate size than did the granules of normal cells. This induced ability to form prominent granules was retained even when the ability to form enlarged cells and to produce colonies was destroyed (Pl. 1, fig. 3). Metachromatic granules were formed in organisms irradiated for as long as 1 hr.

When enlarged organisms were exposed to graduated doses of radiation, the higher doses favoured formation of metachromatic granules (Pl. 1, fig. 4). Excessive irradiation (e.g. for 40 min. with the radiant source at distance of 1 in.) could diminish or completely suppress metachromatic granule formation. There was a corresponding loss of ability to reduce the tetrazoles, potassium tellurite and Janus green B.

Enlarged organisms and prominent granules were produced when irradiated organisms were incubated either aerobically or anaerobically. The visible cytological effects of u.v. irradiation were practically the same for organisms early in the log phase and late in the stationary phase of growth.

Treatment with azaserine also was able to promote the formation of metachromatic granules. Exposure for 1 hr. to 1000 $\mu\text{g./ml.}$ increased metachromatic granule formation; 5000–10,000 $\mu\text{g./ml.}$ were even more effective. In most organisms treated with large doses of azaserine and incubated on agar for as long as 48 hr., prominent metachromatic granules were seen since no normal cells survived to overgrow the agar and obscure the affected organisms.

DISCUSSION

It seems reasonable to conclude that the formation of enlarged organisms and filaments was the result of inhibition of division without proportionate inhibition of growth in organisms which were injured by u.v. radiation or by azaserine. However, it is as yet unknown how specific, if at all, these agents are with respect to inhibition of division. Certainly the division of other bacteria, e.g. *Escherichia coli*, can be inhibited by a great variety of agents.

Metachromatic granules in *Corynebacterium diphtheriae* represent accumulations of metaphosphate (Ebel, 1949; König & Winkler, 1948). These metaphosphate accumulations are formed and disappear, depending on a variety of conditions (Sall, Mudd & Davis, 1955). Their appearance is favoured by the presence of an oxidizable substrate of the tricarboxylic acid cycle and by orthophosphate and potassium. The sites of metachromatic granule formation are in loci possessing redox activity according to Mustakallio & Jännes (1954), who found that these loci in diphtheria organisms reduce Janus green B and the tetrazoles (although Davis & Mudd, 1955, were unable to state this conclusively). Ultraviolet radiation and azaserine may affect these granules by either directly or indirectly altering the phosphate metabolism of the cells. Halvorson (1954), working with aerobically and anaerobically growing yeasts, has shown that the uptake and incorporation of inorganic phosphate into acid-insoluble phosphate is favoured by azaserine.

Apparently the mechanisms involving cell division and viability are more vulnerable to the action of u.v. radiation and azaserine than are the mechanisms concerning metaphosphate accumulation and redox activities of non-metachromatic (and possibly metachromatic) granules.

What common factor or factors in the interaction of bacteria with u.v. radiation and azaserine may underlie such diverse effects as inhibition of

cell division, alterations of phosphate metabolism, mutagenesis, and induction of phage maturation, is of course quite unknown. The fact of the association, however, may suggest some clues for further investigation.

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EXPLANATION OF PLATE

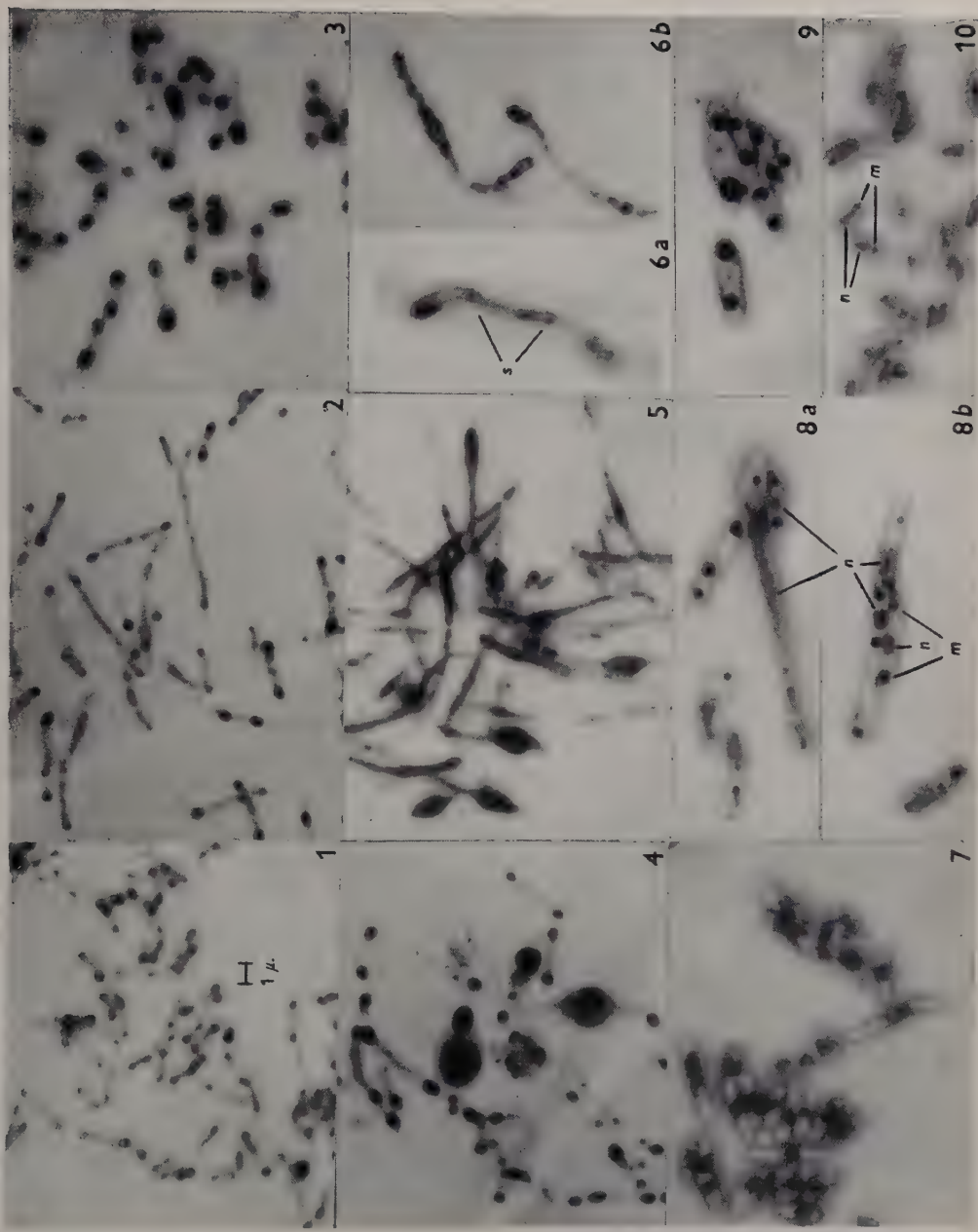
Corynebacterium diphtheriae, strain mitis A9255. Organisms originally grown in blood broth for approximately 18 hr. Organisms lacking metachromatic granules were then exposed to u.v. irradiation at 18.7 in. and subsequently incubated on blood agar.

Fig. 1. Non-irradiated organisms on blood agar for 10 hr. Granules small. Neisser stain.

Fig. 2. Organisms irradiated for 2 min., then on blood agar for 5 hr. After 10 hr. these organisms resemble those in fig. 1. Neisser stain.

- Fig. 3. Organisms irradiated for 15 min., then incubated on blood agar for 10 hr. Neisser stain.
- Fig. 4. Organisms irradiated for 100 sec., then on blood agar for 5 hr. Irradiated cells again irradiated for 80 sec., then incubated on blood agar for an additional 4 hr. With cells only exposed to the first irradiation, enlarged cells and some granulation were evident after 5 hr. However, after 9 hr. these cells resembled normal cells in fig. 1. Apparently the second irradiation promoted granule and enlarged cell formation. Neisser stain.
- Fig. 5. As fig. 4 except stained for nuclear material according to DeLamater using azure A.
- Fig. 6*a*. Organisms irradiated for 100 sec., then on blood agar for 7 hr. followed by 1 hr. on 0.1 % neotetrazolium chloride agar. Cell wall stained according to Hale. S=septa. Other granules are result of reduction of the tetrazole.
- Fig. 6*b*. Numerous sudanophilic granules in cells irradiated 80 sec., then on blood agar for 7 hr.
- Fig. 7. Nuclei in non-irradiated, non-metachromatic cells prior to incubation on blood agar. Modified May-Grünwald stain.
- Fig. 8*a, b*. Organisms irradiated for 100 sec., then on blood agar for 7 hr. Modified May-Grünwald stain. *m*=metachromatic granule; *n*=nuclear material.
- Fig. 9. Organisms irradiated 5 min., then on blood agar 12 hr. Modified May-Grünwald stain. Central, faintly staining areas are nuclear.
- Fig. 10. Non-irradiated organisms on blood agar for 12 hr. Modified May-Grünwald stain. *m*=metachromatic granule; *n*=nuclear material.

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(Facing p. 532)

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Spontaneous Mutation in Stored Spores of a *Streptomyces* sp.

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SUMMARY: Mutations were induced by storing non-dividing asexual spores of a *Streptomyces* sp. at 4° for varying periods of time. Certain stages among spores germinated at 27° were more 'sensitive' than others to this mutagenic action. This high sensitivity was not correlated with particular nuclear stages or with nuclear number. The increased frequency of mutants cannot be attributed to selection (either decreased survival of the wild type or selective growth of previously existing mutants), to increased background (gamma) radiation or to temperature shock. The data indicate that mutation rate is a function of the physiological condition of the spore and that mutations may occur in the absence of nuclear division.

The relative frequency of mutant colonies from stock suspensions of spores of a *Streptomyces* sp. strain T 12 stored in the cold increases with time. It seemed of interest to determine whether this increase was due to a simple selection phenomenon or whether some other factors were responsible.

Streptomyces T 12 was particularly suitable material for this study, because spore suspensions could be readily prepared which consisted almost entirely of uninucleate asexual spores, all in approximately the same physiological condition. These spores showed a high spontaneous frequency of morphological colony mutants (0.5-1.0 %), which were easily scored since they were clearly differentiated from the normal wild-type colony. Large volumes of spore suspensions could be stored for relatively long periods of time (i.e. 3 months) under standard conditions, making it possible to do large-scale experiments under controlled conditions. Under appropriate conditions the effect of storage in the cold could be demonstrated in a relatively short time (20-60 days in the early experiments, 8 days in later experiments).

As described below, the experimental results indicated that as a consequence of storage at 4° new mutational events did, in fact, occur in the spores of *Streptomyces* T 12. These mutations were not a consequence of increases in background gamma radiation, nor was it likely that natural background radiation was inducing the changes. It seemed then that the material might provide some new evidence permitting a discrimination between the two hypotheses: (a) that mutations arise as errors in the duplication of the genetic material (at the time of cell division); and (b) that mutations arise as a consequence of molecular changes caused by mutagenic chemicals within the cells (without the necessity of cell division).

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METHODS

Asexual spores of strain T 12 of *Streptomyces* sp. were used throughout these experiments. All platings were made on a peptone medium composed of: 'Bacto'-asparagine, 0.5 g.; 'Bacto'-peptone, 0.5 g.; K_2HPO_4 , 0.05 g.; glucose, 10 g.; 'Bacto'-agar, 15 g.; and distilled water, 1000 ml.

Fresh spore suspensions were prepared for each experiment. The growth from a single wild-type colony was suspended in water and the surfaces of five peptone agar plates were heavily inoculated with this suspension and incubated at 27°. After 10–14 days the confluent growth on these plates was flooded with cold water, scraped to loosen the spores, decanted, agitated in a 'Waring' Blender in the cold for 1 min. and filtered at 4° through no. 2 Whatman filter-paper to remove any pieces of aerial mycelium (Newcombe, 1953*a*). These suspensions were immediately stored at 4°. Nuclear counts made on stained preparations of these suspensions, using the method described by McGregor (1954), showed them to consist entirely of uninucleate spores and to be almost completely free from clumps of spores.

Spores were aged at 4° in tubes of liquid medium (as above but without the agar) or on the surface of peptone agar on Petri plates. Except where indicated all manipulations of spores to be aged were performed in a cold room at 4°. To determine the frequency of mutants, samples were removed from the tubes, diluted when necessary in distilled water, spread on the surface of agar medium, and incubated at 27° for 6 days. Plates with more than 100 colonies were never used for scoring mutant or viable counts. Where the spores were being aged on the surface of agar, the plates were simply removed from the cold room and incubated at 27° for 6 days. The principal types of mutant colonies scored have been described elsewhere (Newcombe, 1953*a*). In the experiments reported below the majority of mutant colonies were sectorial. As the frequency of whole colony mutants paralleled that of sectorial colony mutants, the results for the two classes are not reported separately. Sectorial colonies were only scored as mutant when the sector obviously originated at the centre of the colony. This discrimination was not difficult since most sectors were obviously peripheral (arising late in the formation of the colony) or central sectors (arising at the time of origin of the colony).

Spores were irradiated with X-rays, using the same techniques as those described by Wainwright & Nevill (1955). In our experiments, the exposure time was 4 min. and the distance, 50 cm.

RESULTS

The effect of ageing spores on agar

Preliminary experiments indicated that storage of a spore suspension of *Streptomyces* T 12 in peptone medium at 4° resulted in an increase in the absolute mutant count upon subsequent plating out. It was decided, therefore, to age the spores on solid medium to determine whether a similar increase in the mutant count could be observed in the absence of any possibility of differential

growth with subsequent fragmentation of the germination tubes during the plating out. Under these conditions, with the spores fixed in position on the plates, an increase in the absolute number of mutant colonies would represent a real increase in the number of mutations and could not be due to selective overgrowth.

A series of 1000 plates was spread with a standard inoculum consisting of 0.1 ml. of spore suspension, and the plates were stored. At various intervals a number of the plates were incubated and subsequently scored for mutant and wild-type colonies; the results of one such experiment are presented in Table 1. At 35 days there was a significant increase in the average number of mutants/plate (0.24–0.36). This increase was even more marked on the 37th day. The results indicated that new mutational events occurred as a consequence of storage and suggested that any similar increases in the proportions of mutants from liquid medium were probably not due solely to selective overgrowth by the mutants.

Table 1. *The frequency of mutants among spores of Streptomyces T 12 stored at 4° on the surface of agar plates*

	Period of storage (days)			
	0	22	35	37
Percentage mutant colonies (no. mutant colonies/total no. colonies)	1.09 (98/8965)	1.64 (57/3479)	2.23 (64/2872)	2.98 (88/2957)
Average no. mutant colonies/plate	0.24	0.29	0.36	0.46
Average no. colonies/plate	21.8	17.8	16.0	15.3
χ^2	—	5.67	19.7	51.3
P	—	0.17	<0.001	<0.001

The χ^2 values given are for comparisons with the 0 day determination of mutant frequency.

Five hundred and thirty-one mutant colonies were sampled and restreaked, and in every case the streaks showed a predominance of mutant colonies, most of which gave rise to further new variant types upon subculture (as described by Newcombe, 1953*a*). The changes studied were then true genetic changes in that they were heritable. Two hundred normal colonies were restreaked and in every case there was a predominance of wild type colonies. Although recombination has been observed in *Streptomyces coelicolor* (Sermonti & Spada-Sermonti, 1955), it has not been observed in this strain T 12. It has therefore not been possible to determine the nature of the mutational event.

The effect of an increased number of nuclei per spore during the period of storage upon the frequency of mutants obtained was tested in the following manner. Eight hundred plates were each spread with a standard inoculum of spores. Four hundred of the plates were immediately incubated at 27° for 6 hr. (so that the average number of nuclei per spore would be *c.* 8) and then returned to 4° to be stored for 33 days along with 200 control plates which had been kept at 4°. The remaining 200 plates were incubated at 27°. The plates which had been preincubated at 27° for 6 hr. before storage showed a marked

increase in both the frequency and the average number of mutants/plate over both the control (0 day) plates and the plates which had been stored in the cold for the same number of days (Table 2).

Table 2. *The frequency of mutants among spores of Streptomyces T 12 stored at 4° on the surface of agar plates*

Period of storage at 4° (days) ...	Period of incubation at 27° before storage at 4° (hr.)		
	0		6
	0	33	33
Percentage mutant colonies	0.91	1.87	4.08
(no. mutant colonies/total no. colonies)	(156/17155)	(85/4552)	(157/3851)
Average no. mutant colonies/plate	0.39	0.42	0.78
Average no. colonies/plate	42.6	22.6	19.2
χ^2	—	29.3	212.4
P	—	< 0.001	< 0.001

The χ^2 values given are for comparisons with the 0 day determination of mutant frequency.

It should be noted that when long preincubation periods were used, the spores developed germination tubes before storage. Since both germinated and non-germinated spores behaved in the same fashion, for convenience the term preincubated spores (or its equivalent) has been used irrespective of the length of the germination tube or the number of nuclei developed.

The effect of varying the period and temperature of preincubation

If the effect of preincubation noted above were a consequence of the increased number of nuclei, the extent of the effect might be expected to vary as the number of nuclei changed. A study was made, therefore, of the effect of varying the period of preincubation at 27° from 0 to 24 hr. upon the mutant frequency of spores subsequently stored on plates at 4° for a standard period of time. Ten ml. samples of a spore suspension diluted in peptone medium at 4° were distributed into 16 × 150 mm. tubes. Half of the tubes were then incubated at 27° by plunging them into a beaker of water at that temperature, agitating briefly and then permitting them to remain in the bath for periods up to 12 hr. For convenience to study the period from 12 to 24 hr. the remaining tubes were held at 4° for 12 hr. and then incubated at 27° for periods of 12–24 hr. When the tubes were removed from the 27° bath, they were plunged into water at 4°, agitated briefly and plated immediately at 4°. Half of the plates at each time interval were immediately incubated at 27°, and the remainder were stored at 4° for 8 days. The results of one such experiment are shown in Fig. 1. The frequency of mutants was not affected by the different preincubation periods (0 day storage) unless the spores were stored at 4° for several days. After 8 days of storage the frequency of mutants rose to a maximum of 26 % for 4½ hr. of preincubation. As the period of preincubation was further increased, the mutant frequency decreased, indicating that sensitivity

to storage was not a simple function of the number of nuclei. The second peak was not as reproducible as the first rise and fall. The maximum mutant frequency for spores preincubated at 27° and stored for 8 days at 4° generally occurred between 4 and 7 hr. of preincubation. However, this was not invariably the case, and the mutant frequencies observed in different experiments for the same conditions of preincubation and of storage are not necessarily entirely comparable (e.g. Fig. 1 and Table 5). Essentially similar results were

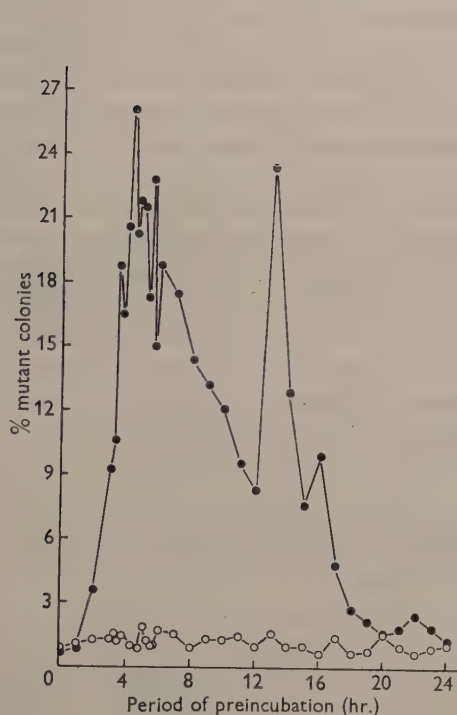


Fig. 1

Fig. 1. The percentage of mutant colonies arising from spores of *Streptomyces* sp. strain T12 stored at 4° for 0 days (○) and 8 days (●) as a function of the period of preincubation of the spores at 27°.

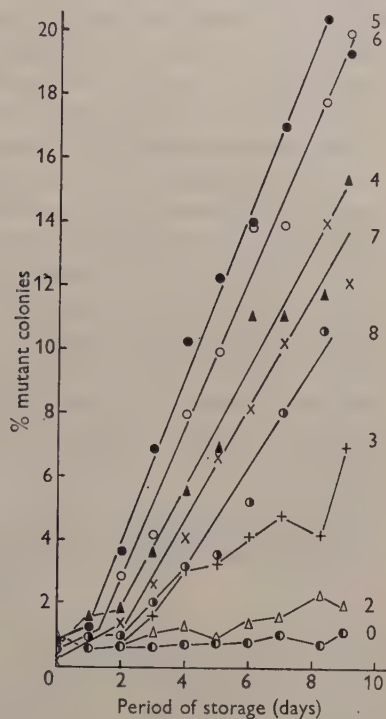


Fig. 2

Fig. 2. The percentage of mutant colonies arising from spores of *Streptomyces* sp. strain T12 preincubated at 27° for 0 hr. (○), 2 hr. (△), 3 hr. (+), 4 hr. (▲), 5 hr. (●), 6 hr. (○), 7 hr. (×), 8 hr. (●); as a function of the length of time of storage at 4°.

obtained with spores which had been preincubated on plates, although the peak occurred approximately 2 hr. earlier. This shift was probably the result of a longer lag in the time for temperature equilibration of the agar plates after the changes.

If a specific nuclear or growth stage were more sensitive to cold storage than others, the temperature at which the spore was grown to reach that stage might be expected not to affect the response. On the other hand, if the physiological condition of the cell were important in determining the response

of the cell to storage, the quantitative response to a given period of storage might be affected by a change in the temperature at which the spores were grown to reach the sensitive stage. A study was therefore made of the effect of varying the temperature of preincubation in peptone medium upon the mutant frequencies attained after subsequent storage under standard conditions. Table 3 presents results from such experiments. The spores preincubated at 27° and 37° showed similar values for the maximum mutant frequencies in different experiments, although the nuclear numbers at the time of these frequencies for the two temperatures were quite different. The peaks at 27° ranged from 15 to 31 % mutants, those for 37° from 16 to 23 %. The maximum for spores preincubated at 27° generally occurred at from 4 to 7 hr. (in one case 9 hr.) of incubation, while at 37° 5 or 6 hr. gave maximal values. Spores preincubated at 18° reached peak mutant frequencies at 7–12 %, and these maxima (after 11 or 12 hr. preincubation) occurred when the number of nuclei were different from those for spores preincubated at 27° and 37°. Thus sensitivity to storage did not correspond to a particular nuclear number or stage, but was rather a function of the physiological state of the spores. Cytological examination at 0 and 8 days for all these experimental suspensions indicated that no further growth of the spores had occurred during the storage at 4° and no changes in the proportion of spores with different nuclear numbers were detected. The rates of increase of mutants on plates stored at 4° after preincubation at 27° for different periods of time are shown in Fig. 2. The rates appeared to differ with different preincubation periods.

Table 3. *The effect of varying the temperature of preincubation of spores of Streptomyces T 12 on the mutant frequency achieved following standard conditions of storage*

Temperature of preincubation (°)	Highest mutant frequency (%)	Nuclear stage, proportion of spores	
		With 4 nuclei (%)	With more than 4 nuclei (%)
18	12	23	7
27	31	51	26
37	23	0	100

The effect of varying the post-storage incubation temperature

An attempt was made to discriminate between the following possibilities: (1) that new mutations occurred and became fixed in the absence of nuclear division; (2) that storage increased the likelihood of mutation (e.g. due to the accumulation of natural mutagen) in the early divisions during incubation at 27° following storage in the cold. If the mutations occurred during storage, the number of mutants observed after a standard storage period might be expected to be largely independent of subsequent growth temperatures. On the other hand, if storage increased the likelihood of mutation in the early divisions after storage, the temperature of incubation after storage might be expected to affect the number of mutants observed.

A study was made to determine whether variation in post-storage incubation temperature altered the mutant frequency. Spores were preincubated for 2 or 4 hr., spread on the surface of peptone agar plates and stored for a standard period. The plates were incubated at 18°, 27° and 37°. The results of three such experiments are shown in Table 4. The only significant deviations from the values for the control plates (incubation at 27°) were those for some spores incubated at 37° (Table 4, columns *d* and *f*). These results, were, however, inconsistent, in that the deviations were in opposite directions, and taken with the results of incubation at 37° (column *b*) would seem to indicate that the changes in post-storage temperature had not affected the mutant frequency.

Table 4. *The effect on mutant frequency of varying post-storage incubation temperature of spores of Streptomyces T 12 stored at 4° on the surface of agar*

Post-storage incubation temperature (°)		Period of storage at 4° (days)					
		(a) 0	(b) 16	(c) 0	(d) 14	(e) 0*	(f) 17*
18	Percentage mutants (no. mutant colonies/total no. colonies)	1.31 (10/764)	14.3 (19/133)	1.42 (64/4494)	24.0 (292/1215)	1.57 (25/1592)	3.52 (32/908)
	χ^2	0.05	0.05	2.22	0.38	0.40	3.06
	<i>P</i>	> 0.8	> 0.8	0.14	0.56	0.5	0.08
27	Percentage mutants (no. mutant colonies/total no. colonies)	1.32 (10/760)	11.1 (21/190)	1.05 (47/4466)	22.9 (314/1370)	1.25 (21/1683)	2.12 (21/999)
37	Percentage mutants (no. mutant colonies/total no. colonies)	0.90 (7/776)	12.0 (16/133)	1.39 (64/4593)	18.0 (159/882)	1.86 (29/1558)	3.70 (36/973)
	χ^2	0.28	0.01	1.91	7.45	1.63	3.93
	<i>P</i>	0.62	> 0.9	0.16	0.006	0.2	0.05

* These spore suspensions were preincubated at 27° for 2 hr. prior to being plated and stored at 4°. The remaining spore suspensions were preincubated for 4 hr.

The χ^2 values given are for comparisons with the corresponding control (27°) determinations of mutant frequency.

The effect of intermittent chilling and warming on the frequency of mutants

All aged spores were subjected to the same number of temperature changes. However, detailed experiments were done to determine whether the spores were more sensitive to temperature shock at some particular stages than at others. A standard spore suspension prepared at 4° was divided into portions which were incubated at 27° in liquid peptone medium for 0 and 2 hr. in one experiment and 0 and 4 hr. in another. Samples from these tubes were plated. Each tube was then chilled at 4° for 15 min., warmed to 27° for 15 min. and

samples were again plated. This cycle of temperature changes was repeated. In addition, samples from a spore suspension were pre-incubated at 27° in peptone medium for 0 and 5 hr., and then stored at 4° for 8 days. Samples were then taken from these latter tubes and plated. The tubes were then subjected to one and two cold-warm cycles as described above. The results (Table 5) showed that no significant change in mutant frequency resulted from the repeated temperature shocks, whether applied before or after the storage period.

Table 5. *The effect on mutant frequency of intermittent temperature changes before and after storage of spores of Streptomyces T12*

Period of preincubation at 27°, no storage (hr.)	No. of cold-warm cycles*	Mutants (%)	No. mutant colonies/total no. colonies	Period of preincubation at 27° followed by storage at 4° for 8 days (hr.)	No. of cold-warm cycles	Mutants (%)	No. mutant colonies/total no. colonies
0	0	1.83	24/1309	0	0	1.20	1/83
0	1	2.30	30/1304	0	1	1.53	13/849
0	2	2.32	30/1292	0	2	1.17	8/864
2	0	1.69	22/1305	5	0	3.73	51/1369
2	1	1.76	23/1309	5	1	3.83	83/2166
2	2	1.19	15/1265	5	2	3.67	64/1746
0	0	1.16	42/3619				
0	1	1.06	40/3778				
0	2	1.19	42/3530				
4	0	0.92	41/4477				
4	1	0.99	36/3631				
4	2	0.84	18/2155				

* Each cold-warm cycle consisted of a period of 15 min. at 4° followed by 15 min at 27°.

The effect of increasing the background radiation

To eliminate the possibility that the increased mutant frequency was due solely, or even in part, to the cumulative effect of any possible slight increases in the background radiation, samples of the same spore suspension were exposed to the gamma radiation emitted by a ⁶⁰Co source. Control spore suspensions shielded from the source were aged (at 4°) in parallel with suspensions which received doses corresponding to $\times 4.4$, $\times 91$, and $\times 810$ the background count. After 11 and 21 days, samples were removed from the tubes and plated to determine the mutant frequency. In addition, after 21 days of storage, samples from both the control tubes and the tubes which had received $\times 810$ the background radiation dose were irradiated with X-rays to determine whether a saturation level had been reached, or whether further mutations could be induced in those stored suspensions. The results (Table 6) indicated that increases in radiation dose far in excess of those encountered as background

radiation did not cause any increase in the frequency of mutants among stored spores. Further, when spores, which had been irradiated at $\times 810$ background during storage, were irradiated with X-rays, an increased mutant frequency was induced (21-day X-irradiated), indicating that the mutations arising during

Table 6. *The effect on mutant frequency (%) of increasing the background radiation during storage of spores of Streptomyces T 12 at 4°*

The spore suspensions were incubated for 4 hr. at 27° before storage at 4°. The radiation source used was ^{60}Co .

No. times background radiation	Period of storage at 4° (days)			
	0	11	21	21*
	Mutant frequency (%) and ratio mutant colonies: total colonies (in parentheses)			
1	1.10 (50/4512)	8.49 (228/2687)	7.68 (63/820)	16.1 (94/584)
4.4	1.02 (43/4223)	7.24 (212/2930)	—	—
91.4	1.11 (48/4338)	7.09 (211/2975)	—	—
810	1.49 (66/4435)	6.16 (206/3346)	7.75 (110/1419)	12.1 (106/873)

* Samples of the 21-day suspension were X-irradiated and plated.

Table 7. *Effect on mutant frequency (%) of increasing the background radiation during storage of spores of Streptomyces T 12 at 4°*

The radiation source used was ^{60}Co .

Period of pre- incubation at 27° (hr.)	No. of times the background radiation	Period of storage at 4° (days)		
		0	3	6
		Mutant frequency (%) and ratio mutant colonies: total colonies (in parentheses)		
4	1	1.18 (23/1945)	9.25 (125/1352)	12.2 (109/893)
	810	1.49 (31/2078)	6.45 (96/1489)	9.45 (103/1090)
	1	1.36 (29/2128)	7.00 (90/1285)	9.13 (48/526)
	810	1.11 (23/2066)	5.01 (67/1338)	7.16 (50/698)

storage were not induced by low intensity radiation and that the cells were still capable of responding to high intensity radiation. The results of a similar experiment, in which samples were plated after 0, 3 and 6 days, are shown in Table 7. These results also show that increased background radiation was not responsible for the increased mutant frequency.

The effects of various other treatments

Spore suspensions, which had been preincubated for 4 or 5 hr. (to bring them to a more sensitive condition), were stored in distilled water at a concentration of c. 500 spores/ml. for a standard period of time. Significant increases in mutant frequency were observed in these suspensions. Limited tests showed that storage of spore suspensions at -14° after freezing still produced significant increases in mutant frequencies. The frequency of mutants following storage was the same whether the spores were stored in continuous light (ordinary incandescent electric bulb) or continuous darkness. The presence of added calcium salts, or of versene during storage was without effect upon the mutant frequency.

DISCUSSION

There have been numerous reports that storage of seeds and pollen (Nava-shin, 1933; Cartledge, Murray & Blakeslee, 1935; Stubbe, 1936; Nichols, 1941), sperm (Kaufman, 1947; Muller, 1946) and bacteria (Kaplan, 1947; Ryan, 1955) has resulted in increases in the frequency of mutations or chromosomal aberrations. However, in general, the results, though suggestive, are equivocal. In some materials (e.g. male *Drosophila*) there is the possibility that additional divisions of genetic material occurred, while in others it has not been possible to eliminate rigorously the alternative of greater survival of spontaneous mutants which were present before storage than of the non-mutants. The observations of Ryan (1955) established that new mutations can occur in non-dividing populations of *Escherichia coli*, maintained in medium exhausted of a limiting growth factor. The present experiments clearly demonstrate that new 'spontaneous' mutational events occurred as a consequence of storage of spores of a *Streptomyces* sp. under conditions where there was no nuclear division, although they do not indicate the nature of the mutational events.

This increased mutation frequency might be a consequence of either mutations which occurred during the storage and without visible nuclear division, or to an increased probability that mutations will occur in the early divisions following storage. It has not been possible to discriminate critically between these two possibilities. However, the results of the experiments in which the post-storage temperature was varied strongly suggest that the former possibility is more likely. The temperature coefficient of the mutation process in *Drosophila* has been observed to be of the order of 3.6–6.5 for a 10° rise in temperature. It seems probable that the mutational event is a chemical reaction, and thus the observed value of unity for the Q_{10} of the processes occurring subsequent to storage, implies that the mutations did, in fact, occur during the storage in the cold. In either event our results are compatible with the hypothesis that mutations occur as errors in the copying of the genetic material. Although no nuclear division was detected in these experiments, the cells may have been metabolizing at a slow rate and duplicated the genetic material without cell division. If this were the case, the number of errors made in this duplication might then be a function of the length of time of storage. On

the other hand, an increased probability of occurrence of an error in gene duplication in the early divisions following storage might be attributed to the accumulation of some mutagens (Newcombe, 1953*b*). The hypothetical mutagen might be a normal metabolic product (Novick & Szilard, 1952), and the amount accumulated would be expected to be dependent upon the length of the period of storage. However, it may be noted that the Q_{10} value of unity obtained for the mutational processes occurring subsequent to storage would require that for stored spores the overall process of gene replication (as distinct from nuclear division) be temperature independent, at least during the first replications after storage.

Influences of the physiological state of the cell on spontaneous mutation have been reported for *Drosophila* (Muller, 1946; Lamy, 1947). Although the time of occurrence of the mutation cannot be critically determined from our experiments, it is of interest to note that the peak of the mutation frequency curve does not appear to correspond to a particular nuclear stage, suggesting that the overall metabolic condition of the spores is of importance in determining the mutagenic effect of storage. If the mutations were occurring during storage, it is clear that they were not associated with a particular nuclear stage but with the physiological condition of the cell. If the mutations were occurring after storage, the mutation rate of spores grown under standard conditions differed according to the growth conditions prior to and during storage and were temperature independent.

I wish to express my thanks especially to Prof. F. J. Ryan for his valuable advice and much encouragement at all stages of this investigation; to Dr H. B. Newcombe for calling my attention to the phenomenon, for his advice and for generously providing facilities; and to J. F. McGregor and A. Nevill for their valuable assistance in different phases of the work. The work described in this paper was performed while the author had a Predoctoral Fellowship from the National Science Foundation, and was submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science of Columbia University.

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Cytochrome c_3 and Desulphoviridin; Pigments of the Anaerobe *Desulphovibrio desulphuricans*

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SUMMARY: Suspensions of various mesophilic strains of *Desulphovibrio desulphuricans* show absorption bands attributable to a cytochrome and a green protein; there are small differences in the position of absorption maxima depending on the strain and culture medium. Both pigments have been extracted, together with flavins rich in flavinadenine dinucleotide; an electrophoretically and chromatographically pure preparation of the cytochrome has been obtained and is designated c_3 . The green protein has been termed 'desulphoviridin'.

Cytochrome c_3 is a soluble autoxidizable thermostable haemoprotein (reduced bands at 553, 525 and 419 m μ .) of low redox potential (-205 mV.), high iso-electric point (pH >10) and containing 0.9% Fe. Degradation studies indicate that it is a bifunctional haemato-haematin with the thio-ether haem-apoprotein links also found in cytochromes c and f ; its m.w. is approx. 13,000 ($S_{20, w} = 1.93 \times 10^{-13}$). Spectroscopic data for various derivatives including haemin c_3 and a porphyrin derivative are recorded. Material purified to at least 94% by cellulose and ion-exchange chromatography acts as carrier in the reduction in hydrogen of sulphite, thiosulphate, tetrathionate or dithionite by detergent-treated bacterial preparations; a similar role has been demonstrated with cell-free systems which reduce sulphite, thiosulphate and tetrathionate. Benzylviologen would replace cytochrome c_3 . No preparation has been obtained showing c_3 -linked sulphate reduction; the evidence for this depends on difference spectra and competition by known sulphate antagonists.

Oxidation of H_2 or organic compounds with O_2 has been demonstrated with these bacteria; the H_2/O_2 reaction takes place fastest in an atmosphere containing 4% O_2 , when oxygen is frequently reduced faster than sulphate. The reaction requires the mediation of cytochrome c_3 and is probably a consequence of the autoxidizability of c_3 .

Desulphoviridin is a thermolabile, soluble, acidic porphyrinoprotein absorbing at 630, 585 and 411 m μ .; no metabolic function has been detected. It is stable over a limited pH range and decomposes readily, yielding a chromophoric group which fluoresces red in ultraviolet light, absorbs at 595 m μ in neutral and alkaline solution (solution red) and at 612 m μ in acid solution (solution blue-green). This material can be purified by chromatography on 'Florisil' or paper. It is very photo-sensitive and water-soluble. Its character is obscure; it may be a highly carboxylated chlorin. Spectroscopic data are recorded.

The cytochromes have for long been regarded as pigments characteristic only of aerobic or facultatively anaerobic bacteria. For many years they were believed to be absent from obligate anaerobes (see Keilin, 1933; Keilin & Slater, 1953), a view which was supported by a recent examination of seven anaerobes, which allowed for the possibility of adaptive cytochrome formation in air (Schaeffer & Nisman, 1952). However, a cytochrome has now been observed in the sulphate-reducing bacterium *Desulphovibrio desulphuricans* (Butlin & Postgate, 1953; Ishimoto, Koyama & Nagai, 1954*a, b*; Postgate,

1954*a, b*), though this bacterium is an exacting anaerobe (Grossman & Postgate 1953*a, b*); cytochromes have also been demonstrated in species of the obligately anaerobic photo-autotrophs *Chromatium* and *Chlorobium* (Kamen & Vernon, 1954*a, b*; Vernon & Kamen, 1954; Gibson & Larsen, 1955). The present paper describes the spectroscopic properties of a strain of sulphate-reducing bacteria, the extraction of the components responsible for the spectroscopic absorption bands, the purification of the cytochrome component, and some of its chemical and biological properties.

METHODS

Spectroscopy. The behaviour of suspensions of bacteria was observed in a Hartridge reversion spectroscope modified for use with relatively opaque material and calibrated with the emission lines of neon; quoted readings were taken at limiting dilution as recommended by Lemberg & Legge (1949). Spectrophotometric measurements were made in the Hilger 'Uvispek' instrument. Measurements on intact organisms were made by the procedure of Barer (1955), in which scattering was reduced to a minimum by suspending the organisms in a medium of refractive index similar to that of themselves. The refractive index of the bacteria used in this work was shown by phase-contrast microscopy to be 1.383 ± 0.001 ; therefore a solution of bovine serum albumin (Armour Laboratories, Fraction V) of c. 34 % (w/w; refractive index, measured in the hand refractometer, equivalent to 31.5 % sucrose) was the most suitable suspending medium.

Manometry. Conventional Warburg manometers were used for experiments involving gas exchanges; details of procedure were given by Grossman & Postgate (1955).

Organisms used and their cultivation. *Desulphovibrio desulphuricans* strain Hildenborough (National Collection of Industrial Bacteria NCIB 8303), purified according to Postgate (1953) was used, except where otherwise mentioned; its origin, maintenance and methods of subculture were described by Postgate (1951*a*). Large quantities of organisms for fractionation were obtained from continuous culture experiments being conducted elsewhere in this laboratory (Report, 1953); the conditions of culture changed in detail from time to time, but normally the bacteria were grown in a medium containing Na_2SO_4 equivalent to 0.4 % (w/v) S, yeast extract (Difco) 0.4 % (w/v) and small amounts of NH_4^+ , K^+ and Mg^{++} (Report, 1954, p. 53). Effluent containing 0.4–0.8 mg. air-dry wt. organisms/ml. was harvested during several days, centrifuged in 100 l. lots in a Sharples centrifuge, the organisms washed with distilled water and dried by addition to 10 vol. cold acetone (4°), followed by washing with acetone and then ether. Yield: 30–35 g. dried material/100 l. culture medium. Organisms freshly harvested during the logarithmic phase of growth were used for metabolic experiments; a few experiments were done with bacteria from batch cultures.

Chromatography. Cellulose columns were packed with Whatman cellulose powder (standard grade) and washed with 4 vol. of distilled water. Ammonium

'Amberlite' columns were prepared as follows: a finely divided form of Amberlite IRC 50 (XE 97, Chas. Lennig and Co. Ltd.) was obtained and the fraction which sedimented between 3 and 20 min. in water was collected. This was converted to the ammonium form by adding excess 2 N-NH₄OH, packed and washed with distilled water (c. 8 vol.) until the effluent contained less than 0.0025 N-HN₄OH (pH 9.5 to 10). Mean flow rate: 75 ml./hr. The resin XE 97 was also used for chromatography with sodium phosphate buffers (0.34 g.-ion Na⁺/l.) following the procedure described by Boardman & Partridge (1955). It was washed successively with 2 N-NaOH, 2 N-HCl, then water and buffer; the columns (12 × 0.9 cm. diam.) were then packed and run at room temperature; mean flow rate 0.8–1.1 ml./hr. Florisil (30/60 mesh; Floridin Co., Florida, U.S.A.) was packed in water and washed with 20 vol. of 0.1 N-HCl followed by 20 vol. distilled water. Cross-linked polymin P columns were prepared following the advice of Mr D. K. Hale of this Laboratory. Polymin P (Badische Anilin und Soda Fabrik, Germany; a soluble polyethylenimine; 40 g.) was mixed with 40 ml. methanol, 4 ml. epichlorhydrin and 36 g. powdered cellulose (above) in that order; this mixture was allowed to polymerize at 60° for 3 days, and the product broken up in 2 N-HCl and washed successively with water, 2 N-NaOH, water, 2 N-acetic acid and 0.02 N-sodium acetate buffer (pH 5.0). The column, after packing, was washed with distilled water. Paper chromatography was conducted at room temperature on Whatman no. 1 filter-paper sheets, using the ascending method.

Electrophoresis. Electrophoresis on strips of Whatman no. 1 paper was carried out in the EEL instrument (Evans Electroselenium Ltd., Harlow, Great Britain).

Potentiometry. The Cambridge pH meter was used for fine pH measurements with a glass electrode, and for potentiometric titrations with a bright Pt electrode and a calomel reference electrode. Redox potentials were studied by titrating the test material in KH₂PO₄ (1 %, w/v, adjusted to pH 7 ± 0.02 with 2 N-NaOH) against Na₂S₂O₄ (30 mg./ml. buffer) in a current of O₂-free N₂ in a thermostat at 30 ± 0.05°.

Units of cytochrome. The millimolar extinction coefficient of the cytochrome studied here is not known. Evidence is presented in this paper that the cytochrome has a similar molecular weight to cytochrome *c* but two haemin groups per molecule. Hence ϵ_{mM} should theoretically be 54 (compare 27 for cytochrome *c*); the units used in this paper are derived from spectrophotometric measurements at the α -peak in the reduced form: 1 mUnit (mU.) = 1 mMole if $\epsilon_{\text{mM}} = 54$.

Abbreviations. The following abbreviations are used in this paper: CTAB for cetyltrimethylammonium bromide, FAD for flavin adenine dinucleotide, FMN for flavin mononucleotide, DPN for diphosphopyridine nucleotide, TPN for triphosphopyridine nucleotide, ATP for adenosine triphosphate, TCA for trichloroacetic acid.

RESULTS

Observations with whole bacteria

Suspensions of the Hildenborough strain heavier than 10 mg. dry wt./ml. KH_2PO_4 (0.5%, w/v; pH 6.9) showed strong adsorption bands at 553 and at 630 $\text{m}\mu$. when viewed through a depth of 0.5 cm. A weaker band at 525 $\text{m}\mu$. was seen, and old suspensions showed a shading about 595 $\text{m}\mu$. This last band was later shown to be due to a chromophore liberated by partial decomposition of the 630 $\text{m}\mu$.-component. The bands at 525 and 553 $\text{m}\mu$. disappeared on

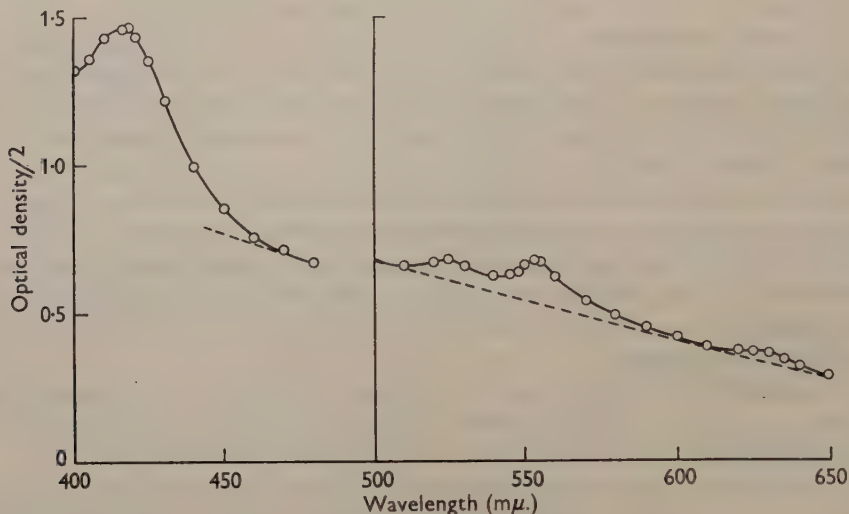


Fig. 1. Absorption spectra of *Desulphovibrio desulphuricans* (Hildenborough). Organisms were suspended in strong bovine plasma albumin and examined spectrophotometrically through a depth of 0.5 cm. after addition of $\text{Na}_2\text{S}_2\text{O}_4$. Visible range: 65 mg. dry wt./ml.; Soret range: 20 mg./ml. Dotted lines represent hypothetical scatter curve of a suspension of bacteria devoid of pigments.

shaking in air and returned on (a) standing, (b) passing in H_2 , (c) adding $\text{Na}_2\text{S}_2\text{O}_4$. The band at 630 $\text{m}\mu$., and the 595 $\text{m}\mu$. band when present, were not affected by these procedures, nor by addition of H_2O_2 , $\text{K}_3\text{Fe}(\text{CN})_6$, or on passing in pure O_2 . The bands at 525, 553 and 630 $\text{m}\mu$. intensified and shifted to shorter wavelengths on freezing and de-vitrifying a suspension in 50% glycerol with liquid N_2 , but remained single; hence they represented single compounds (Keilin & Hartree, 1955). Inspection of the suspension through a blue filter (dilute methylene blue) showed that the limit of visibility in the violet shifted from about 420 $\text{m}\mu$. to about 410 $\text{m}\mu$. with aerated suspensions. The absorption bands were recorded spectrophotometrically by Barer's procedure (Fig. 1).

This sulphate-reducing organism thus contained a reversibly oxidizable pigment resembling the cytochrome *c* of muscle, responsible for absorption peaks at 553 and 525 $\text{m}\mu$. as well as at about 420 $\text{m}\mu$. in the reduced condition. The

absorption peak at 630 $m\mu$., though similar in position to cytochrome a_2 , had none of the properties of a conventional cytochrome. The cytochrome probably represented the sole intracellular haematin, because treatment of a suspension of bacteria with $\text{Na}_2\text{S}_2\text{O}_4$ in the presence of alkali and pyridine led to a shift of the cytochrome band to about 550 $m\mu$., but no increase in its intensity; hence no additional haemochromogens were formed.

The presence of cytochrome and the 630 $m\mu$.-component was confirmed with five other mesophilic strains of *Desulphovibrio desulphuricans* examined in washed suspension at 8–12 mg. dry wt. organisms/ml. (Table 1). Small differences in the position of the bands occurred between strains, wider variation occurred with bacteria derived from different media: El Agheila Z had its strongest band at 555 $m\mu$. when harvested from lactate media and at 559 $m\mu$. from malate media (for media see Grossman & Postgate, 1955). This was not due to the presence of another or a different cytochrome, since: (a) cytochrome extracted from malate-grown organisms by the procedure described below had its α -peak at the usual 553 $m\mu$.; (b) the α -band in malate-grown organisms was homogeneous in liquid N_2 .

Table 1. *Visible absorption spectra of suspensions of various strains of Desulphovibrio desulphuricans*

Bacteria were harvested from lactate media except in the case indicated when El Agheila Z was grown in a malate medium. All strains halophilic except those marked *.

Strain	NCIB no.	Absorption peaks ($m\mu$.)		
		525	553.8	630.0
Hildenborough*	8303	525	553.8	630.0
California 43:63	8364	525	555.2	630.0
El Agheila Z	8380	525	555.2	630.8
El Agheila Z (malate)	—	525	559.8	629.0
Canet 41	8393	525	553.6	630.0
Wandle*	8305	525	555.7	630.0
Venice 2	8323	525	554.0	628.5

Oxidation by sulphate

It was possible that the cytochrome was concerned as a carrier in the reduction of sulphate and other reducible anions (see Postgate, 1951*b*), but addition of sulphate or sulphite to washed suspensions in anaerobic conditions led to no obvious change in the visible absorption bands. This was not surprising, however, since the end product of reduction of these compounds—sulphide—is a powerful reducing agent and might well mask any oxidation. To avoid this difficulty, suspensions of bacteria were incubated *in vacuo* in Thunberg tubes containing CdCl_2 (10 %, w/v) in the side arm to remove sulphide continuously, and, after 30 min. at room temperature at pH 7.0, the visible cytochrome bands were clearly less intense, by some 40 %, in the presence of excess sodium sulphate, sulphite, thiosulphate or tetrathionate, than in a control suspension without these. The peak at 630 $m\mu$. did not alter in these conditions, except that, after prolonged exposure to sulphite, evaporation of SO_2 produced conditions sufficiently alkaline to cause decomposition, as a result of which

a band appeared at 595 $m\mu$, and the suspension fluoresced red in ultraviolet light. Ishimoto, Koyama & Nagai (1954*b*) reported observation of cytochrome oxidation by certain sulphur-containing anions without taking these precautions; the discrepancy between our results may be attributable either to the use of different strains or to difference in the amount of sulphide present at the moment of adding the oxidizing agent.

The oxidation of cytochrome by sulphate was recorded by difference spectra (Fig. 2). Since the modified spectrophotometric apparatus recommended by Chance (1954) was not available, Fig. 2 was obtained with suspensions of organisms rendered translucent by Barer's procedure. The introduction of sulphate caused a slight change in the refractive index of the suspending medium as compared with the control; in consequence the difference spectrum in Fig. 2 did not lie exactly about the abscissa.

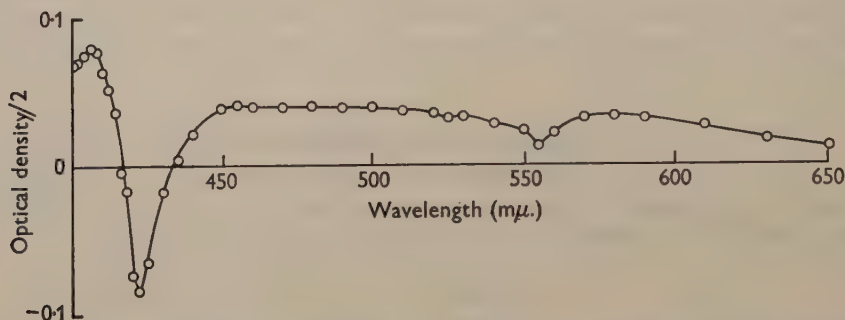


Fig. 2. Difference spectrum of *Desulphovibrio desulphuricans* (Hildenborough). The absorption of a bacterial suspension (7.2 mg. dry wt./ml. plasma albumin) containing excess sulphate was measured with a reference cell containing a similar suspension without sulphate.

Effect of inhibitors

Selenate or monofluorophosphate, which are competitive inhibitors of sulphate reduction (Postgate, 1949, 1952), inhibited the oxidation of the cytochrome by sulphate for several hours, though they did not affect oxidation by sulphite. The absorption bands were unaffected by passing in pure CO, adding KCN (10^{-2} M), Na_2S (5×10^{-3} M), or NH_2NH_2 (10^{-2} M); their reaction with air took place normally in the presence of these compounds. CTAB (recrystallized from acetone; 100 $\mu\text{g.}/\text{mg.}$ dry wt. organisms) inhibited the reduction of the oxidized cytochrome on standing, but did not prevent its reduction in H_2 .

Mode of action of CTAB

Salton (1951) showed that CTAB rendered Gram-positive aerobes permeable to substances of low molecular weight. Suspensions of *Desulphovibrio desulphuricans* treated with CTAB (100 $\mu\text{g.}/\text{mg.}$ dry wt.) were killed: the viable count by the method of Grossman & Postgate (1953*b*) decreased from $1.7 \times 10^9/\text{ml.}$ to $2.4 \times 10^3/\text{ml.}$ Material absorbing at 265 $m\mu$. was demonstrated in the supernatant liquid after centrifugation together with free cytochrome,

though hydrogenase and the 630 m μ -component were absent. Thus CTAB rendered the organisms permeable to the cytochrome; this conclusion was supported later by the observation that untreated bacteria reduced added purified cytochrome only slowly in H_2 , though CTAB-treated organisms reduced it practically instantaneously. CTAB-treated organisms did not reduce sulphate in H_2 , though they reduced methylene blue at a normal rate; they also did not reduce methylene blue with lactate or pyruvate.

Extraction of the pigments and purification of the cytochrome

The routine procedure given below for extraction of the cell pigments was devised to give maximum yields, as well as preparations of hydrogenase and of denatured cytochrome; it is therefore more complicated than if cytochrome only had been required and if yield had been unimportant. A rapid procedure by which purified cytochrome alone may be obtained in about 24 hr. is also given.

Routine procedure. Acetone-dried organisms were extracted at 4° with KH_2PO_4 (0.5 %, w/v; pH 7.0 ± 0.1) yielding a green-brown solution containing cytochrome, 630 m μ -component, hydrogenase, free flavins and flavoproteins. The latter were removed by addition of H_2SO_4 (2 N) to pH 5.0 ± 0.2 and the precipitate extracted with NH_4HCO_3 , dialysed in H_2 and freeze-dried to yield the hydrogenase fraction. The supernatant fluid from the precipitation at pH 5 was half saturated with $(NH_4)_2SO_4$ and the precipitate, containing the 630 m μ -component and some cytochrome, was extracted with NH_4HCO_3 (c. 1 %, w/v), dialysed, passed through a column of ammonium Amberlite-XE 97 to remove as much cytochrome as possible, dialysed and freeze-dried as the 630 m μ -component. Some purified cytochrome was eluted from the Amberlite column with NH_4OH (0.25 N) and added to the major fraction obtained later. The supernatant from the $(NH_4)_2SO_4$ precipitation was brought to pH 2.6 ± 0.2 with 2 N- H_2SO_4 and the precipitate, whose cytochrome content increased the longer it was left in contact with the supernatant, was dissolved in $NaHCO_3$ solution and dialysed. The supernatant fluid was passed through a column of Whatman 'standard grade' cellulose powder, the effluent (containing flavins) discarded and the adsorbed cytochrome eluted, together with some flavin, with NH_4OH (0.25 N) and dialysed. The combined dialysed products from the last two steps were adsorbed on to a column of ammonium Amberlite-XE 97 and washed with water. The coloured effluent contained denatured cytochrome which was collected and freeze-dried; it had no enzymic activity and reacted with CO. The native protein was eluted with NH_4OH (0.25 N), dialysed and freeze-dried. Fig. 3 records the steps in a typical preparation. Sulphuric acid was used in preference to TCA for pH adjustment because some earlier fractionations with TCA had yielded wholly denatured products—consistent with Lewis's (1954) observation that TCA has a more drastic effect than H_2SO_4 on haemoproteins. Verhoeven & Takeda (1956) encountered similar difficulties during the extraction of bacterial cytochrome c from nitrate-reducing bacteria, and finally chose citric acid for their fractionation procedure. Filtrations were

avoided at all stages since the cytochrome was readily absorbed on cellulose; Keilin & Hartree (1945) reported that salt-free solutions of cytochrome *c* behaved similarly. Ishimoto & Koyama (1955) used an essentially similar procedure based on acetone precipitation in place of acid fractionation.

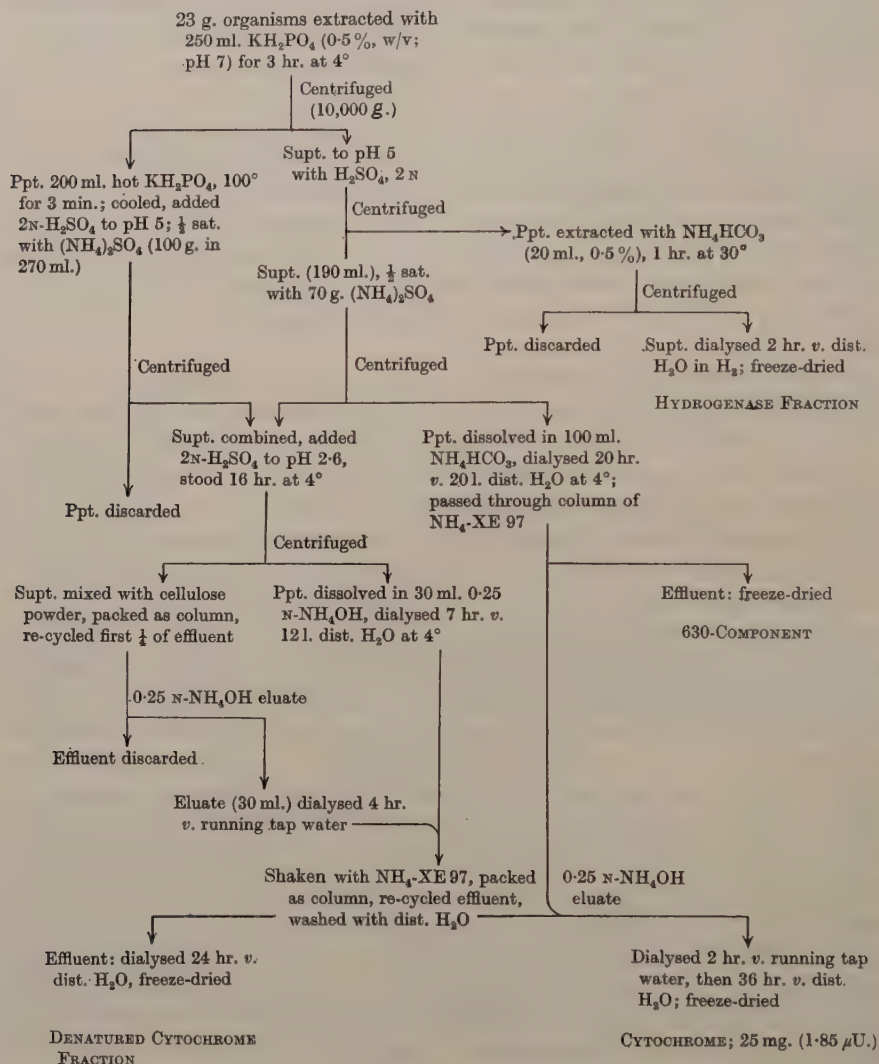


Fig. 3. Fractionation of acetone-dried *Desulphovibrio desulphuricans* (Hildenborough). The bacteria contained *c.* 0.22 μU . cytochrome c_3 /g. air-dry mass; assuming their acetone-dried mass was similar, the yield was *c.* 28 % of theory.

Rapid procedure. Rapid procedures based on the release of cytochrome by CTAB or by heat were sometimes used; a record of a typical preparation follows: wet centrifuged bacteria (28 g. dry wt./100 ml. distilled water) were added slowly to 1 l. boiling and stirred KH_2PO_4 solution (0.5%, w/v;

pH 7.0 ± 0.05), boiled 3 min. and allowed to cool at 4° . After 4 hr. the mixture was centrifuged, the supernatant fluid dialysed for 16 hr. against 12 vol. distilled water and shaken with 50 ml. ammonium Amberlite XE 97, packed as a column, and the first portion of effluent re-cycled to ensure complete absorption of cytochrome. As in the earlier procedure, a portion of 'denatured' material, able to react with CO, was not held by the resin. The pink column was washed with distilled water, the cytochrome eluted with $0.25\text{ N-NH}_4\text{OH}$, and the product dialysed against 100 vol. distilled water and freeze-dried. The yield was 16.6 mg. purified powder (c. 19% theory).

THE PROPERTIES OF THE CYTOCHROME OF *DESULPHOVIBRIO DESULPHURICANS*

A preliminary account of the chemical and physical properties of the bacterial cytochrome has appeared elsewhere (Postgate, 1955*b*).

Spectrum. The cytochrome prepared by the procedure above had typical haematin spectra (Fig. 4). The analogy of the ferricytochrome spectrum at pH 7.0 to that of ferricytochrome *c* type III (Theorell & Akesson, 1941) is

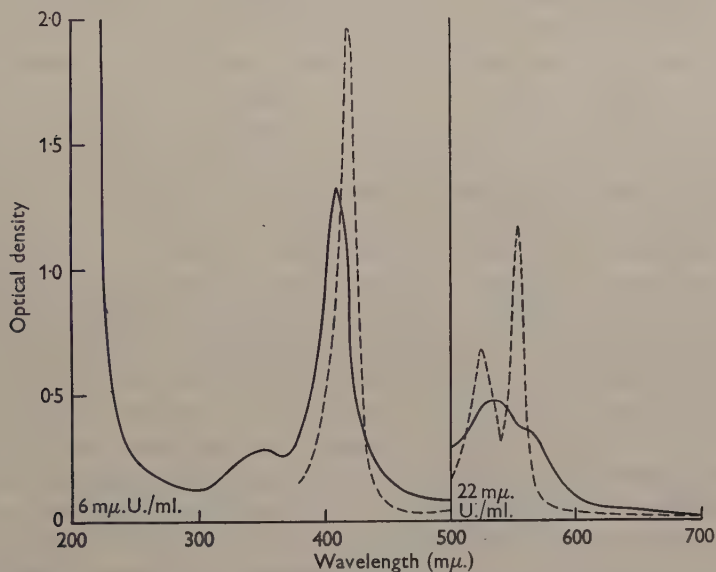


Fig. 4. Spectrum of purified cytochrome c_3 from *Desulphovibrio desulphuricans* (Hildenborough). Readings were taken at 1 mμ. intervals at absorption peaks, 5 mμ. intervals elsewhere; pH 7.00 ± 0.02 ; $t = 20^\circ$; 22 mμU. cytochrome/ml. in visible range, 6 mμU./ml. in ultraviolet; - - -, reduced form; —, oxidized form.

marked, even to the inflexion about $565\text{ m}\mu$., but the band at $280\text{ m}\mu$. was missing and no evidence for a band about $685\text{ m}\mu$. (Theorell, 1948) was found. The ferrocytochrome spectrum was not examined in the ultraviolet region since it was not stable in the absence of dithionite, which absorbs in this region, but the visible and Soret peaks again resembled those of cytochrome *c*

except that they were shifted a few $m\mu$. towards the red. The visible peaks were measured with greater precision in the optical spectroscope and were $\alpha = 553.2 m\mu$. and $\beta = 525.2 m\mu$. at pH 7.00 ± 0.02 and 19° . Some ratios of the intensities of various peaks are given in Table 2.

Table 2. *Ratios of heights of absorption peaks in cytochrome c_3*

$\alpha = 553 m\mu$., $\beta = 525 m\mu$., $\gamma = 419 m\mu$., $\delta = 350 m\mu$..

Peaks	Ratio
$\gamma : \alpha$ (reduced)	5.75
$\gamma : \alpha$ (oxidized)	12.6
γ (reduced) : (oxidized)	1.51
$\gamma : \delta$ (oxidized)	6.6
$\alpha : \beta$ (reduced)	1.52

The specific extinction coefficient was obtained by dissolving known amounts of well-dialysed, freshly chromatographed cytochrome in buffer; at 20° and pH 7.0 $\epsilon_{sp.} = 4.20 \pm 0.06$ in the reduced condition at $553 m\mu$., and 1.60 in the oxidized condition at $535 m\mu$.

Purity. Electrophoresis on paper in KH_2PO_4 (0.5 %, w/v, pH 7.0) showed only the single cytochrome zone even after staining with bromothymol blue. Chromatography in buffer on a cation exchange resin (Boardman & Partridge, 1955) indicated small amounts of a colourless impurity which absorbed light at $280 m\mu$.; the pH value of the buffer used was less than pH 7.0 recommended by Boardman & Partridge for cytochrome c , since at the latter pH value the band due to the bacterial cytochrome spread badly. A typical experiment showing the presence of impurity absorbing at $280 m\mu$. is recorded in Fig. 5; the R_f value of the impurity was 0.9–1.0 (that of the cytochrome was 0.38), and a rough integration indicated that it amounted to less than 6 % of the total protein. Since purification by chromatography in buffer was laborious and conveniently applicable only to small amounts of material, the work reported in this paper was done with material containing this impurity.

Reactions in solution. The freeze-dried powder had a deep red colour. It dissolved completely in phosphate buffer or 0.25 N- NH_4OH to give a clear solution; it also dissolved in distilled water but much more slowly, and salt-free solutions were best prepared by dialysis of salt-containing solutions. Preparations stored for over 1 month at -12° sometimes contained insoluble brown material, but the supernatant fluid was enzymically active. Exposure of solutions to preparations of organisms caused some denaturation, since cytochrome recovered from metabolic experiments (below) always contained a portion of material which was not retained by ammonium Amberlite XE 97, and which reacted with CO.

No spectroscopic change occurred in any of the following conditions: heating at pH 7.0 to 100° for 5 min.; passing in pure CO; adding KCN up to $10^{-2} M$; adding excess ascorbic acid, $NaBH_4$, $K_4Fe(CN)_6$ or $FeSO_4$. With cysteine the reduced form of the cytochrome appeared slowly and incompletely, with Na_2S it appeared slowly but completely, and with $Na_2S_2O_4$ it appeared at once. Sodium nitrite and diluted acetic acid gave a nitroso-derivative of the

oxidized form (visible bands of equal intensity at 532.5 and 563.5 $m\mu$.; compare 531.1 and 563.4 $m\mu$. for muscle cytochrome c); treatment with 2 N -NaOH denatured the preparation, since it then reacted with CO (absorption peaks: 415, 530, 565 $m\mu$.; compare 415, 531.5, 564.5 $m\mu$. for muscle cytochrome c) or with pyridine + dithionite (absorption peaks of 'pyridine] haemochromogen':

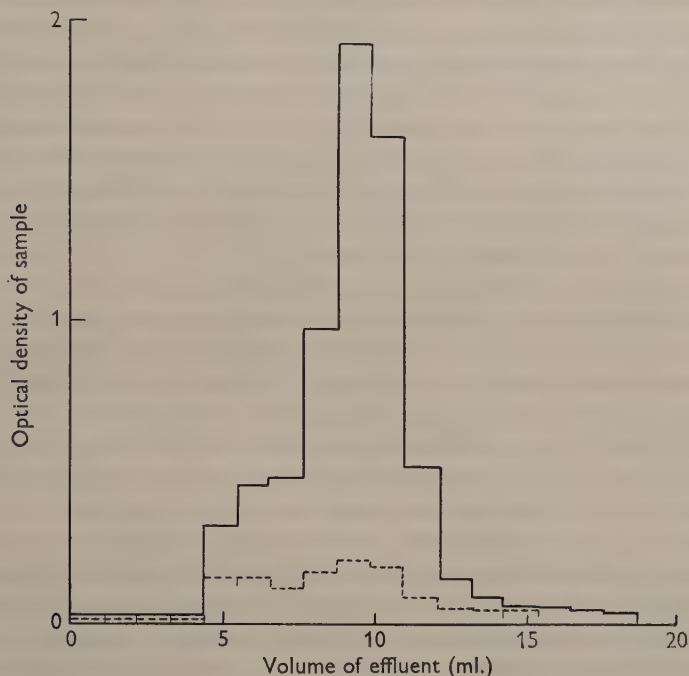


Fig. 5. Chromatography of purified cytochrome from *Desulphovibrio desulphuricans* (Hildenborough). 2 mg. cytochrome c_3 was chromatographed in phosphate buffer (pH 6.40 ± 0.02) on Amberlite XE 97 (see text), flow rate 1.1 ml./hr.; room temperature; retention volume of column 3.8 ml. Absorptions of 1.1 ml. samples at 410 and 280 $m\mu$. (dotted lines) were plotted; R_f of cytochrome c_3 (410) = 0.38.

413, 521.5, 551.8 $m\mu$.; compare 415, 521.8, 550.6 $m\mu$. for muscle cytochrome c). The denatured form had no enzyme activity, and its reduced absorption spectrum at pH 7.0 had bands at 418, 552.6 and 524.9 $m\mu$. which were indistinguishable from the native protein without special care.

Autooxidizibility. The cytochrome was apparently autooxidizable, but it was desirable to prove that this was a property of the native protein since apparent autooxidizibility might also be due to denaturation or contamination with an oxidase. The following evidence is in favour of the view that the native protein is autooxidizable: (i) enzymically active preparations were fully autooxidizable but did not react with CO; (ii) the oxidation was not affected by KCN (10^{-2} M), a powerful inhibitor of cytochrome c oxidase; (iii) no sign of a cytochrome c oxidase was detected at any stage in the purification of the cytochrome; (iv) the standard redox potential (below) was such that, on physico-chemical grounds, the reduced form was unlikely to be stable in air.

Iso-electric point. The fact that the cytochrome could be purified by ion-exchange with an ammonium Amberlite XE 97 suggested a basic iso-electric point; this was confirmed by paper electrophoresis in KH_2PO_4 buffers (0.5 %, w/v) adjusted to various pH values with 20 % (w/v) NaOH, on paper strips of 5 cm. width with the instrument setting at 2 mA./strip (potential between buffer compartments about 380 V.). At pH 6.99, 8.10, 9.23 (borate buffer) and 10.15 the protein migrated towards the cathode rather more slowly than chromatographically purified cytochrome *c* on the same strips. At pH values in the region of ten paper strips took up CO_2 with a decrease in pH value, so that a precise determination of the iso-electric point was not attempted. However, during a 2 hr. run starting at pH 10.66 (the iso-electric point of cytochrome *c*) and ending at pH 10.30 the protein migrated slowly towards the cathode together with a control spot of cytochrome *c*. Hence the iso-electric point probably lies between these values.

Redox potential. Careful reduction with $\text{Na}_2\text{S}_2\text{O}_4$ in the presence of redox dyes indicated a standard potential between that of sodium indigo disulphonate ($E'_0 = -125$ mV.) and benzylviologen ($E'_0 = -395$ mV.) and lying in the range of Janus green ($E'_0 = -225$ mV.); Ishimoto *et al.* (1954*b*) obtained a similar value with their preparation. This strongly reducing potential accounted for the failure of ascorbic acid, $\text{K}_4\text{Fe}(\text{CN})_6$, etc., to reduce the protein, and also made impracticable the measurement of the standard redox potential by spectrophotometric procedures (e.g. Davenport & Hill, 1952) since there is no stable redox system in this range. The potential was therefore determined by potentiometric titration in O_2 -free nitrogen with excess anthraquinone-2-sulphonic acid ($E'_0 = -250$ mV.), which did not absorb significantly in visible light and which poised in the appropriate potential range. Three titrations of 0.15 μU . cytochrome + 1.5 mg. anthraquinone-2-sulphonic acid in 29 ml. sodium phosphate buffer (0.34 g. ion Na^+/l .; pH 7.0 ± 0.02) at $30 \pm 0.3^\circ$ against $\text{Na}_2\text{S}_2\text{O}_4$ indicated a standard potential of $E'_0 = -205 \pm 4$ mV.

Molecular weight. The sedimentation coefficient of a preparation of the cytochrome was determined by Dr A. G. Ogston at the Department of Biochemistry, University of Oxford. A solution of *c*. 0.5 % cytochrome in NH_4OH (0.25 M), dialysed overnight against 1000 vol. of a solution containing NaCl (0.1 M) + Na_2HPO_4 (0.0627 M) + KH_2PO_4 (0.0133 M) had a sedimentation coefficient $S_{20, w} = 1.93 \times 10^{-13} \pm 2$ % (compare 1.83×10^{-13} quoted for cytochrome *c* of 0.43 % Fe content; Paul, 1952). The sedimenting boundary seemed symmetrical, no obvious sign of heterogeneity was observed, and all the light-absorbing material appeared to be associated with the sedimenting material. This sedimentation coefficient implies a minimum possible value of 10,200 for the molecular weight, on the assumption that $f/f_0 = 1$, the particles being spherical, unhydrated and with a hydrodynamic specific volume equal to the partial specific volume taken as 0.71, the value given by Theorell (1936) for cytochrome *c*. Since most globular proteins have values of f/f_0 considerably greater than 1, the sedimentation coefficient above would be consistent with a molecular weight in the region of 13,000 (see Discussion).

Iron content. Analysis of some residues recovered from metabolic experi-

ments indicated a high iron content (0.75 %), and a single analysis of 8.1 mg freshly chromatographed, well-dialysed cytochrome, by the *o*-phenanthroline procedure (Sandell, 1944) indicated an iron content of 0.92 %.

Linkage of prosthetic groups. The haemin of cytochrome *c* is linked to the apoprotein by thio-ether links to the porphyrin ring, as well as by co-ordination to the iron atom; as a result the molecule shows considerable thermo-stability and resistance to cleavage of its prosthetic group from the apoprotein by acids. Moreover, the molecule readily yields, with mineral acids, an ether-insoluble porphyrin in which the sulphur-containing amino acid groups remain attached to the porphyrin residue.

The bacterial cytochrome showed similar thermal and acid stability. Porphyrin was released from the oxidized form only by concentrated H_2SO_4 (bands at 404, 550.8, 593 $\text{m}\mu$. in 2 N-HCl; HCl number, 0.05 ± 0.01 %); the reduced form was less stable and 3.3 N-HCl in the presence of $\text{Na}_2\text{S}_2\text{O}_4$, released mainly a porphyrin which was insoluble in ether + glacial acetic acid (15 %, v/v), and which had its main band at 554 $\text{m}\mu$. (compare 553 $\text{m}\mu$. quoted by Keilin (1933) for 'porphyrin *c*').

Ice-cold acetone containing 10 % (v/v) glacial acetic acid, which removes the haemin from haemoglobin, catalase, etc. (see Lewis, 1954), precipitated the bacterial cytochrome unchanged. Ice-cold acetone containing 1 ml. 5 N- H_2SO_4 /50 ml. acetone did not yield an ether-soluble haemin, although the protein was converted to a brown water-soluble material from which ether-soluble haemin could be obtained by Paul's (1950) procedure. These properties all suggest that thio-ether links participate in the binding of prosthetic groups and protein in the bacterial cytochrome.

Nature of prosthetic groups. A haemin was released from the cytochrome by Paul's (1950) procedure. A typical experiment was as follows: 1 ml. chromatographed cytochrome solution (0.7 $\mu\text{U}/\text{ml}$.) was treated with 0.2 ml. glacial acetic acid and 1 ml. AgSO_4 (8 mg./ml.) for 30 min. at 75–80°, cooled and the haemin extracted three times into ether containing 25 % (v/v) acetic acid, dried, dissolved in KH_2PO_4 (0.5 % w/v, pH 7.0) and examined spectrophotometrically. The product had a Soret band at 390 $\text{m}\mu$. as compared with 391 $\text{m}\mu$. found in a control preparation of haematohaemin from cytochrome *c*. Pyridine haemochromes prepared from these haemins were spectroscopically closely similar: pyridine haematohaemochrome had peaks at 409, 518.5 and 546.0 $\text{m}\mu$.; the pyridine haemochrome found from the isolated haemin of the bacterial cytochrome absorbed at 408, 517.2 and 546.0 $\text{m}\mu$. It is interesting that, as with cytochrome *c*, the spectrum of the 'pyridine haemochromogen' formed directly from the protein (quoted earlier, p. 555) differed from that obtained from the separated haemin.

These observations suggested that the prosthetic groups of cytochrome *c* and the bacterial cytochrome were closely similar, and further evidence for this was obtained by reductive fission of the protein to porphyrin. Davenport (1952) obtained mesoporphyrin and a chlorin from cytochrome *c* by reduction *in vacuo* with sodium amalgam; degradation of the bacterial cytochrome in this manner gave a porphyrin spectroscopically resembling mesoporphyrin as

well as a chlorin (α -band at 643 m μ . in ether, identical with a control preparation from cytochrome *c*). The wavelengths in m μ . of the absorption peaks of the porphyrin, freed of chlorin, in dioxane and in 2 N-HCl are given below:

In dioxane	Mesoporphyrin from cytochrome <i>c</i>	402, 496.5, 529.8, 566.8, 621.5
	Porphyrin from bacterial cytochrome	402, 497, 530.2, 566.5 621.5
In 2 N-HCl	Mesoporphyrin from cytochrome <i>c</i>	402, 547.5, 570.5, 590.5
	Porphyrin from bacterial cytochrome	402, 549.2, 571, 591

These observations permit the tentative conclusion that the prosthetic groups of the bacterial cytochrome are OH-substituted haematohaemins linked to the protein by thio-ether bridges as in cytochrome *c*, a view further supported by the close spectroscopic similarities of the pyridine, carboxy- and nitroso-derivatives of the two proteins (p. 555).

Metabolic function of the bacterial cytochrome

The probability that the cytochrome acted as electron carrier during the biological reduction of sulphate and related ions was investigated further by studying preparations of bacteria able to oxidize the cytochrome anaerobically with these substrates and by measuring the effect of the cytochrome on the rates of substrate reduction in hydrogen by these preparations. Owing to shortage of cytochrome the quantitative aspects of its effect on these reactions could not be studied thoroughly, but some qualitative impressions of its relative activity are recorded. A preliminary account of this work was given elsewhere (Postgate, 1955*a*).

Anaerobic cytochrome oxidation

Bacteria treated with CTAB (50 μ g./mg. dry wt.) oxidized the cytochrome anaerobically with Na₂SO₃, Na₂S₂O₃ or Na₂S₄O₆, but not with Na₂SO₄, provided precautions were taken to remove sulphide continuously as in the earlier experiments with living organisms (above). In a typical experiment a suspension of 0.2 mg. CTAB-treated cells/ml. at pH 7.0 was placed in a double side-arm Thunberg tube under H₂ until all added cytochrome (5 m μ U./ml.) was reduced. One side arm contained CdCl₂ (10 %, w/v) on filter-paper to absorb sulphide, the other contained substrate. The hydrogen was then pumped out, the substrates added *in vacuo* and the suspension examined spectroscopically. The intensities of the cytochrome bands, compared with controls without substrate or with Na₂SO₄, diminished markedly during 5 min., though the bands did not disappear entirely even after several hours.

While this work was in progress, Millet (1955) obtained a cell-free sulphite reductase from another strain of *Desulphovibrio desulphuricans*, and kindly provided instructions on how to prepare active extracts. Such preparations, made from the Hildenborough strain, contained, in addition to a sulphite reductase and hydrogenase, thiosulphate and tetrathionate reductases. Being

transparent they permitted quantitative measurement of the extent of cytochrome oxidation by sulphite, thiosulphate and tetrathionate (Table 3). A constant percentage oxidation was reached much more slowly with Na_2SO_3 than with $\text{Na}_2\text{S}_2\text{O}_3$ or $\text{Na}_2\text{S}_4\text{O}_6$; quoted values in Table 3 are corrected for a slow oxidation which occurred in the control tubes without substrate.

Table 3. *Anaerobic oxidation of cytochrome c_3 by cell-free extracts of Desulphovibrio desulphuricans (Hildenborough)*

Vacuum-dried organisms were shaken with distilled water (25 mg./ml.) for 1 hr. at 37° under N_2 and the debris removed at 15,000 g (15 min.). The supernatant fluids were diluted 1/3 with KH_2PO_4 (0.5 %, w/v; pH 7.0 ± 0.05) containing 25 μU . cytochrome/ml., added to double side-arm Thunberg tubes containing substrate in one arm and CdCl_2 (10 %, w/v) on filter-paper in the other. After reducing the cytochrome in H_2 the substrates were added *in vacuo* and the optical density at 554 $m\mu$. was measured at intervals until a constant percentage oxidation was reached.

Substrate	expt:	Percentage oxidation of cytochrome	
		1	2
Na_2SO_3		19	14
$\text{Na}_2\text{S}_2\text{O}_3$		70.5	76
$\text{Na}_2\text{S}_4\text{O}_6$		82	84

Substrate reduction in hydrogen

CTAB released cytochrome from the bacteria. Consequently, cytochrome-linked reactions would be expected to be inhibited by CTAB owing to dilution of the co-factor, and the inhibition should be overcome by adding large amounts of purified cytochrome. This approach was used to investigate the effect of cytochrome on the reduction of substrates in hydrogen.

Thiosulphate reduction. CTAB (50 $\mu\text{g.}/\text{mg.}$ dry wt. organisms) prevented the reduction of sulphate in H_2 by bacterial suspensions, but permitted slow reduction of sodium thiosulphate. Addition of the cytochrome augmented the reaction rate (Fig. 6), thus indicating a carrier action. FAD, DPN, TPN and ATP had no carrier action.

Tetrathionate reduction. Similar experiments, using sodium tetrathionate in place of thiosulphate, showed that the cytochrome acted as a carrier for the tetrathionate reductase system (Fig. 6).

Sulphite reduction. Similar experiments, with sulphite in place of thiosulphate, showed that the cytochrome acted as a carrier in this system, but its effect was much less marked than with thiosulphate and tetrathionate (Fig. 6). This may be due to: (a) a greater requirement for cytochrome in this system; (b) need for additional co-factors; (c) greater damage to the sulphite reductase caused by CTAB. The latter explanation was favoured by the further observations that doubling the CTAB concentration totally inhibited the sulphite reductase, though the thiosulphate reductase remained active. A mixture of FAD, DPN, TPN and ATP did not augment the effect of the cytochrome.

Dithionite reduction. Sodium dithionite hydrolyses in anaerobic solution to a mixture of thiosulphate and metabisulphite, hence the ability of the

cytochrome to act as carrier in its reduction by CTAB-treated bacteria (Fig. 6) was not unexpected. The experiment is of interest however, since it demonstrated that cytochrome augmented the reaction rate in an environment having, at least initially, a redox potential of about -350 mV. (that of a weak dithionite solution), some 150 mV. more reducing than the standard potential of the cytochrome itself.

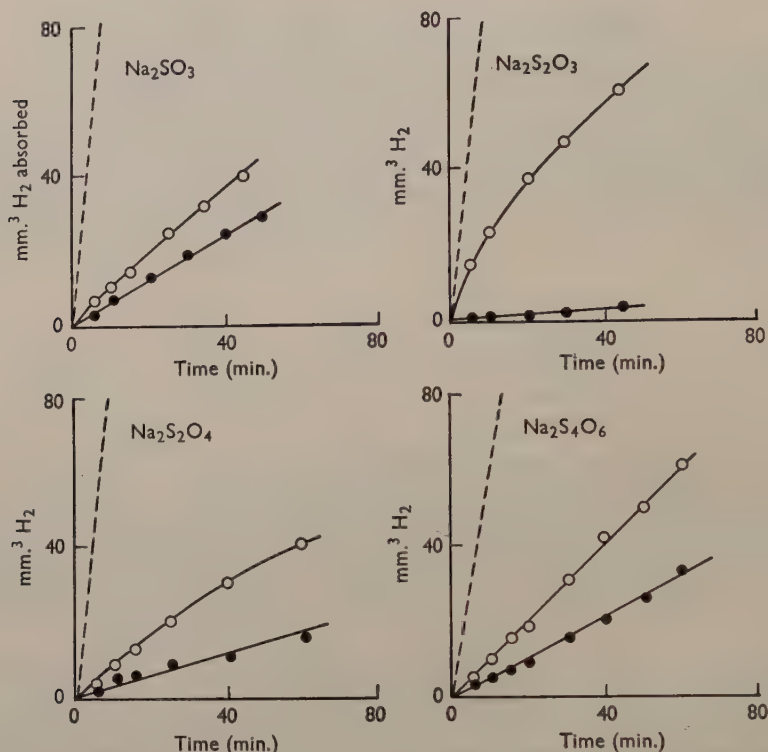


Fig. 6. Cytochrome-linked reductases in CTAB-treated *Desulphovibrio desulphuricans* (Hildenborough). Organisms (1.5 mg./vessel) were treated CTAB (50 μ g./mg. dry wt. bacteria) and their ability to reduce substrates in H_2 with and without cytochrome c_3 was measured manometrically. Cytochrome: 50 m μ U./ml.; vessels preincubated 1 hr. before adding c. 1 mg. substrate (dithionite added as solid); centre well: $CdCl_2$ (0.25 ml., 10 %, w/v); reaction fluid 1.25 ml. final; pH 6.9 ± 0.05 ; 37° , H_2 gas phase; dotted lines indicate average rates of substrate reduction without CTAB, O; cytochrome; ●, without added cytochrome.

Sulphate reduction. The cytochrome did not influence the rate of reduction of sulphate in H_2 by intact bacteria. None of the following preparations reduced sulphate in hydrogen: organisms treated with CTAB, acetone-dried or vacuum-dried bacteria and soluble extracts therefrom; extracts of cells ground with Al_2O_3 ; hence experiments analogous to those recorded above were not undertaken. The failure of acetone-dried bacteria to reduce sulphate conflicts with a report by Sadana & Jagannathan (1954), who stated that sulphate acted as hydrogen acceptor for crude preparations of acetone-dried organisms. Indirect

evidence pointing to a function in sulphate reduction is summarized later (see 'Discussion').

Oxygen reduction. The fact that the cytochrome is autoxidizable gave theoretical reasons for suspecting that the sulphate-reducing bacteria could reduce oxygen in spite of their anaerobic habit; synthesis of water from H_2/O_2 mixtures was detected and reported earlier (Postgate, 1954*b*). The reaction took place at a maximum rate with H_2 /air mixtures containing 4% (v/v) O_2

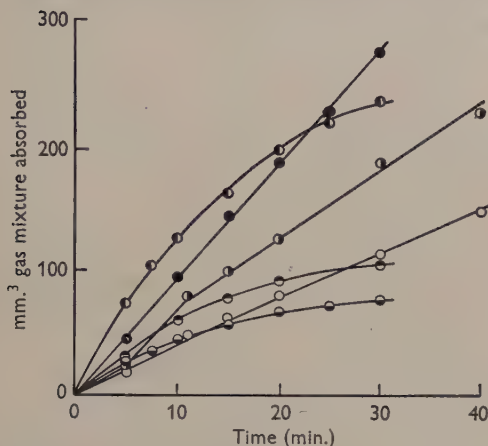


Fig. 7

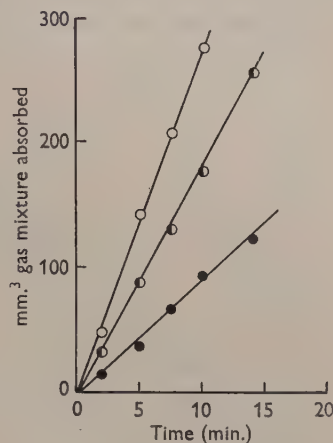


Fig. 8

Fig. 7. Hydrogen-oxygen reaction in *Desulphovibrio desulphuricans* (Hildenborough). Bacteria were harvested from a lactate yeast extract + sulphate static culture and the rates of gas uptake in hydrogen-air mixtures were determined manometrically. 9.25 mg. dry wt. organisms/vessel; total fluid volume 3 ml.; buffer: KH_2PO_4 (0.5%, w/v; pH 6.3 ± 0.05); 37° , gas phase concentrations of O_2 (v/v): \circ , 1%; \bullet , 2% \bullet , 4%; \circ , 8%; \bullet , 12%; \bullet , 16%.

Fig. 8. Effect of cytochrome c_3 on hydrogen-oxygen reaction by CTAB treated *Desulphovibrio desulphuricans* (Hildenborough). Bacteria were treated with CTAB (100 μg /mg. dry wt.) and the rate of gas uptake measured manometrically. 4.3 mg. dry wt. organisms/vessel; fluid volume 1.5 ml.; buffer: KH_2PO_4 (0.5%, w/v; pH 6.9 ± 0.05). \circ , without CTAB; \bullet , with CTAB; \bullet , with CTAB + cytochrome c_3 (250 $m\mu U$ /ml.).

(Fig. 7); at lower pO_2 values availability of oxygen presumably limited the reaction velocity, and at higher pO_2 values oxidation of hydrogenase inhibited the reaction. This situation is familiar in the biological aerobic oxidation of hydrogen, but the system in *Desulphovibrio desulphuricans* appears to be more sensitive to oxygen inhibition than most (compare optimal pO_2 values of 8% for *Hydrogenomonas flavus*, Kluver & Manten, 1942; 4–10% for *Azotobacter vinelandii*, Wilson, Lee & Wilson, 1942; about 9% for *Escherichia coli*, Lascelles & Still, 1946; 5–15% for an unspecified *Hydrogenomonas* sp., Schlegel, 1953).

A remarkable feature of the H_2/O_2 reaction was that, in the optimum atmosphere of 4% (v/v) O_2 , oxygen was frequently reduced faster than sulphate. The Q_{O_2} of the control curve in Fig. 8 is -129 mm.³/mg. dry wt. organisms/hr., corresponding to an oxygen reduction rate of 5.8 μ mole/mg./hr.

The mean Q_{H_2} value in several experiments with sulphate as hydrogen acceptor was $-440 \text{ mm.}^3/\text{mg./hr.}$, corresponding to a sulphate reduction rate of $4.9 \text{ } \mu\text{mole/mg./hr.}$

CTAB ($100 \text{ } \mu\text{g./mg.}$ dry wt. organisms) decreased the reaction velocity to a low value, and addition of cytochrome increased the reaction rate again (Fig. 8). Oxidation of lactate, pyruvate, fumarate and malate with O_2 as terminal H-acceptor ($N_2 + 4 \text{ } \%$ (v/v) O_2) was demonstrated with strain Hildenborough (Postgate, 1954*b*) and strain El Agheila Z (Grossman & Postgate, 1955), but was not studied further.

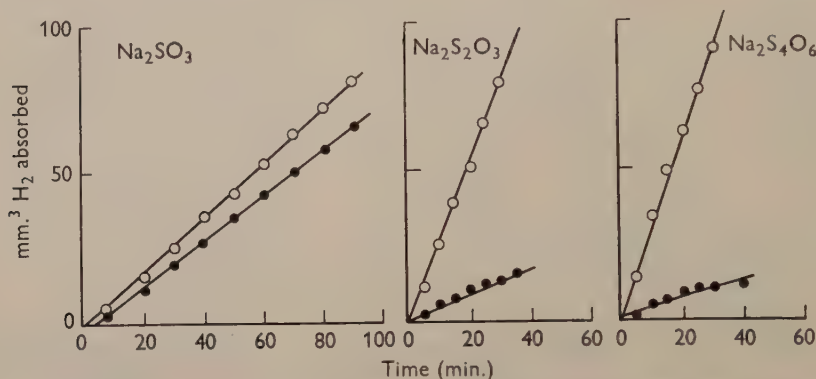


Fig. 9. Influence of cytochrome c_3 on hydrogen uptake by cell-free reductase preparations from *Desulphovibrio desulphuricans* (Hildenborough). Vacuum-dried bacteria were extracted under N_2 at 37° with distilled water (1 ml./25 mg.; 1 ml./50 mg. for sulphite reductase) and the supernatant fluids (0.5 ml.) used in the manometers. Fluid volume 1.5 ml.; buffer: KH_2PO_4 (0.5 %, w/v; pH 6.3 ± 0.05); centre well: $CdCl_2$ (0.25 ml., 10 %, w/v); substrates: $5 \text{ } \mu\text{mole}$; H_2 gas phase; 37° . O, with cytochrome c_3 ($560 \text{ m}\mu\text{U./ml.}$; $680 \text{ m}\mu\text{U./ml.}$ with sulphite reductase); ●, control without cytochrome.

Reduction by cell-free preparations. The demonstration of cytochrome-linked reductases by the use of CTAB was open to the criticism that the cytochrome may act, not as an electron carrier, but merely by reversing chemically an inhibitory effect of CTAB. It was thus desirable to demonstrate carrier action by a technique not involving CTAB.

Extracts of vacuum-dried bacteria prepared following Millet's advice reduced thiosulphate, tetrathionate or sulphite in hydrogen, unlike extracts of acetone-dried cells, which reduced only thiosulphate in these conditions. The reduction rate was augmented by addition of the bacterial cytochrome (Fig. 9). As in the experiments with CTAB-treated organisms, the effect of the cytochrome on $Na_2S_2O_3$ and $Na_2S_4O_6$ reduction was much more marked than on Na_2SO_3 reduction. The reason for this seemed most likely to be that the total sulphite-reductase content of the extracts was low since benzylviologen, which also has a cytochrome-like effect on the sulphite reductase system, did not much augment the activity of these preparations.

Cytochrome-like effect of benzylviologen. Ishimoto, Koyama & Nagai (1955) extracted a soluble thiosulphate-reductase system from their strain of *Desulphovibrio desulphuricans* which conducted the reaction: $S_2O_3^{2-} + H_2 = SO_3^{2-} + H_2S$,

and whose reaction rate was augmented by benzyl- or methyl-viologen. Later Ishimoto & Koyama (1955) showed that their bacterial cytochrome had a similar effect on these preparations. It was of interest to see whether benzylviologen had a cytochrome-like effect on the major systems examined in the present work. Suspensions of CTAB-treated bacteria reduced benzylviologen with $\text{Na}_2\text{S}_2\text{O}_3$, $\text{Na}_2\text{S}_4\text{O}_6$ or Na_2SO_3 , but not with Na_2SO_4 . In a typical experiment a suspension of 0.33 mg. CTAB-treated bacteria/ml. at pH 7 was treated as in the comparable experiments with the bacterial cytochrome except that benzyl viologen ($2 \mu\text{mole/ml.}$) replaced the added cytochrome. The time taken to decolorize the reduced benzylviologen at 37° was noted. In contrast to

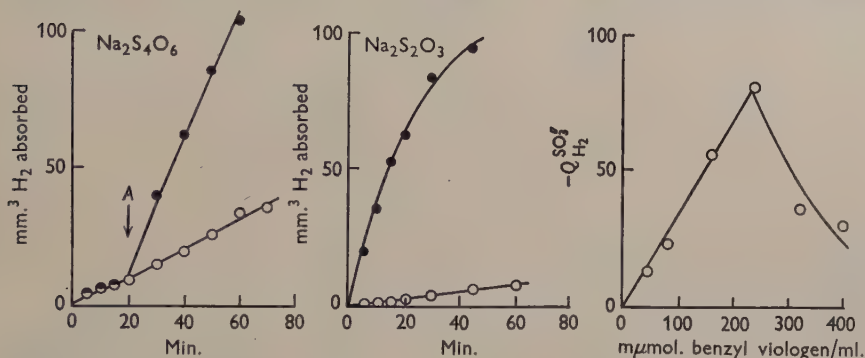


Fig. 10. Cytochrome-like effect of benzyl viologen. Organisms of *Desulphovibrio desulphuricans* (Hildenborough) were treated with CTAB ($50 \mu\text{g./mg. dry wt.}$) and the rates of hydrogen uptake in the presence of reducible substrate measured manometrically. Cell contents as for Fig. 6 except that benzylviologen replaced cytochrome c_3 . With sulphite as substrate a curve illustrating the effect of benzylviologen concentration on substrate reduction rate is given; with tetrathionate the benzylviologen was tipped in after the start of the experiment (A). ●, with $200 \text{ m}\mu\text{mol}$ benzylviologen/ml.; ○, control.

their behaviour with cytochrome, the suspensions re-oxidized reduced benzyl viologen completely with the substrates mentioned; re-oxidation with Na_2SO_3 was markedly slower (70 min.) than with thiosulphate (15 min.) or tetrathionate (8 min.); without substrate or with Na_2SO_4 the dye remained reduced for more than 170 min. Jebb (1949) used a somewhat similar technique to show that the 'tetrathionase' of a coliform organism re-oxidized reduced Nile blue. Benzylviologen augmented the rate of reduction of thiosulphate, tetrathionate or sulphite in hydrogen by CTAB-treated bacteria and by cell-free reductase preparations. A selection of curves illustrating this is given in Fig. 10; the findings confirm and extend those of the Japanese workers.

THE COMPOUND ABSORBING AT $630 \text{ m}\mu$.

Concentrates of the material prepared as described always contained variable amounts of cytochrome and material insoluble in water. The material was purified further by extracting into distilled water and passing the solution through a column of cellulose coated with an aliphatic polyimine resin in the

acetate form (see 'Methods'). The effluent contained cytochrome and flavo-protein, and the 630 $m\mu$.-component remained on the column as an emerald green zone. It was eluted with sodium acetate + acetic acid buffer (5 M, pH 5.0) and dialysed against distilled water.

Properties. After prolonged dialysis, preparations of the 630 $m\mu$.-component precipitated in the dialysis sac, but the precipitate re-dissolved, on adding traces of phosphate buffer or NaHCO_3 , to give an emerald-green solution. This solution had the spectrum shown in Fig. 11; the Soret peak at 411 $m\mu$. did not change in height or position on adding dithionite, indicating that the material was free from cytochrome; there was an inflexion at *c.* 390 $m\mu$., a minor peak at 585 $m\mu$. and a strong peak at $632.5 \pm 0.2 m\mu$. Paper electro-

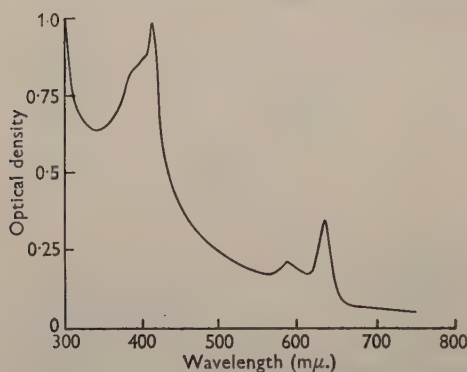


Fig. 11

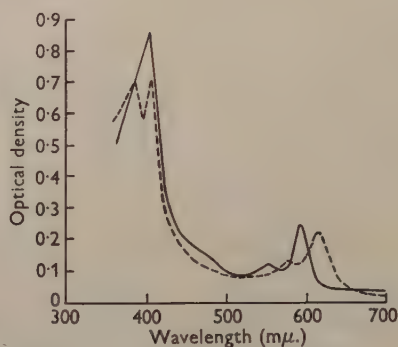


Fig. 12

Fig. 11. Spectrum of chromatographed '630 $m\mu$.-component'. Readings taken at 1 $m\mu$. intervals at the Soret peak, 2.5 $m\mu$. at α -peak, 5 $m\mu$. at β -peak, 10 $m\mu$. elsewhere. Protein in KH_2PO_4 (0.5 %, w/v; pH 7.0 ± 0.05); 20°.

Fig. 12. Spectra of chromophore from '630 $m\mu$.-component'. Spectra obtained after chromatography on 'Florasil' (see text). —, in acetic acid (0.1 N); ---, in HCl (N). Readings every 1 $m\mu$. at Soret peaks, 2.5 $m\mu$. at visible peaks, 10 $m\mu$. elsewhere.

phoresis showed an iso-electric point on the acid side of pH 7.0. On heating above 70° or treatment with acid to pH < 4 or alkali to pH > 9 the 630 $m\mu$.-band shifted towards the green and the solution fluoresced red in ultraviolet light (365 $m\mu$.). No evidence for formation of a pyridine haemochrome was obtained; the main visible band moved to 595 $m\mu$. with alkali with or without pyridine and/or dithionite.

Chromophoric group. The red fluorescent material was readily photo-oxidized with loss of fluorescence, and had to be handled in the dark, where it survived 3 min. in contact with warm conc. H_2SO_4 . It was slightly soluble in ether containing 15 % (v/v) glacial acetic acid, but was not extracted quantitatively from aqueous solution with this mixture; it returned to the aqueous phase on shaking with distilled water. It was chromatographed in the dark on paper in 5 % (w/v) Na_2HPO_4 , running as a single spot of $R_f = 0.74$ (20°). The chromophore was also adsorbed as a blue-green band from acid solution by 'Florasil' and was eluted with aqueous pyridine (1 % v/v) as a pink-brown solution with

an intense red fluorescence; with dilute mineral acids the colour changed to blue-green and the fluorescence became more purple. The spectra of preparations obtained in this fashion depended on the pH value of the solution (Fig. 12). Both acid and neutral forms showed their major visible absorption peak in the red, unlike a simple aetioporphyrin; the absorption peaks lay at 404, 594.5 and 551.0 $m\mu$. (alkaline or neutral) and 385, 404, 575.0 and 613.5 ($N-HCl$). The position and height of the double Soret peak of the acid form were unchanged in 2 $N-HCl$. A neutral spectrum of this material was not successfully obtained owing to its insolubility in organic solvents.

The chromophore was also observed by its fluorescence in old cultures that had become alkaline owing to loss of H_2S from the medium. Red fluorescence in u.v. light after adding NaOH to a culture was a sensitive test for the presence of the 630 $m\mu$ -component in various strains. The chromophore was undoubtedly responsible for the shading at 595 $m\mu$, sometimes observed in the spectrum of washed bacterial suspensions.

OTHER PIGMENTS

The spectrum of crude extracts of acetone-dried organisms showed a hump at about 450 $m\mu$, which disappeared on adding dithionite or on passing in hydrogen; simultaneously the extracts ceased to fluoresce yellow in ultraviolet light, a change characteristic of flavins. Protein was removed from such extracts with TCA (4 %, w/v), the TCA was removed by extraction of the tri-*n*-octylamine salt into chloroform (Hughes & Williamson, 1951), and the extracts concentrated and chromatographed in the dark on paper in *n*-butanol/acetic acid/water (4 : 1 : 5) or aqueous Na_2HPO_4 (5 %, w/v). The chromatograms indicated the presence of FAD, FMN and traces of riboflavin. An examination of freshly harvested *Desulphovibrio desulphuricans* was undertaken by Dr J. L. Peel, who showed that the Hildenborough strain has a low flavin content compared with most other anaerobes he has examined, but an unusually high ratio of FAD to FMN; the riboflavin observed was probably an artefact of the acetone-drying procedure; Dr Peel's results are reported elsewhere (Peel, 1955). As would be expected, these flavins exist in the normal cell as conjugates, since CTAB at concentrations able to release cytochrome into the medium released no flavin. No evidence was obtained for association of flavin with the cytochrome, unlike the cytochrome b_2 -lactic dehydrogenase system of Appleby & Morton (1954).

DISCUSSION

The cytochrome. Table 4 lists the properties of a number of cytochromes which have an α -peak within 1 $m\mu$. of that of the cytochrome described here, and shows that it is not identical with any of them. It may be identical with a degradation product of heart muscle cytochrome *b* (Hübscher, Kiese & Nicolas, 1954), but insufficient published data are available to judge. Hence the systematic name '*Desulphovibrio desulphuricans* cytochrome 553' should be applied to the material studied here (see Scarisbrick, 1947), but since this

name is cumbersome and does not bring out its relation to muscle cytochrome *c*, the trivial name of cytochrome *c*₃ has been adopted.

The relationship to cytochrome *c* may be summarized: *c*₃ is thermostable, soluble, has firm chemical linkings between the haemin and apoprotein and is strongly basic. It differs mainly in (a) its low redox potential and consequent autoxidizibility; (b) its different metabolic function; (c) details of its spectrum.

Table 4. *Some properties which distinguish various cytochromes from that present in Desulphovibrio desulphuricans ('cytochrome c₃')*

Pigment	Reference	Source	Major distinctive properties
Cytochrome <i>c</i> ₁ (<i>e</i>)	1	Heart muscle	Thermolabile, not autoxidizable
Cytochrome <i>f</i>	2	Green leaves	Not autoxidizable, positive E'_0
Cytochrome <i>b</i> ₄	3	Halotolerant bacterium	Not autoxidizable, acid iso-electric point
' <i>Chlorobium limicola</i> cytochrome 553'	4, 5	<i>C. limicola</i>	Not autoxidizable, positive E'_0
' <i>Acetobacter suboxydans</i> cytochrome 554'	6	<i>A. suboxydans</i>	Insoluble, not autoxidizable
'Hemoprotein 554'	7	Heart muscle	Positive redox potential
' <i>Chromatium</i> ' cytochrome	8	<i>Chromatium</i> 'D'	High m.w., limited pH stability
Cytochrome <i>c</i> ₃	—	<i>D. desulphuricans</i>	Thermostable, low m.w., soluble, autoxidizable, negative E'_0 , basic iso-electric point

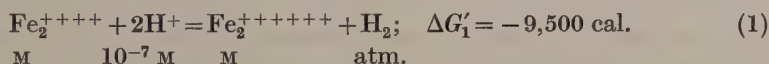
References: 1, Keilin & Hartree (1955); 2, Davenport & Hill (1952); 3, Egami *et al.* (1953); 4, Kamen & Vernon (1954*a*); 5, Gibson & Larsen (1955); 6, Smith (1954), also personal communication; 7, Widmer *et al.* (1954); 8, Newton & Kamen (1955).

A further difference from cytochrome *c* may be noted. The specific extinction coefficient of ferrocytochrome *c* at 550 m μ . is about 2.1, whereas $\epsilon_{sp.}$ for cytochrome *c*₃ at 553 is about double this (4.2). Since the molecular weights of cytochromes *c* and *c*₃ are of a similar order, it follows that *c*₃ has two haemin groups/molecule. This view is confirmed by the high iron content (0.92 %) of *c*₃, more than twice that of the purest *c* recorded (see Paul, 1952).

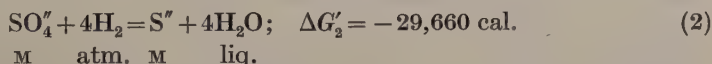
Though *c*₃ can act as carrier in reactions in which oxygen is terminal hydrogen acceptor, the strictly anaerobic character of these bacteria emphasized by Grossman & Postgate (1953*a*) leaves little doubt that these reactions are of limited metabolic significance and merely reflect the low redox potential of *c*₃. Such reactions might, however, provide a mechanism by which the organism could remove traces of O₂ from its environment since O₂, while not lethal, prevents growth and sulphate reduction. The absence of inhibition by KCN, CO, etc. indicates that no ordinary cytochrome *c* oxidase is present, and the presence of a cytochrome *c* oxidase insensitive to cyanide like that of *Myrothecium verrucaria* (Darby & Goddard, 1950) is excluded since neither suspensions nor preparations of the strain studied here oxidized reduced cytochrome *c*. However, though no preparation has been obtained in which a linkage between cytochrome *c*₃ and sulphate reduction could be demonstrated directly,

difference spectra, the effect of sulphate antagonists, and the demonstration of a role in the reduction of sulphite (the one established intermediate; Millet, 1955) makes such a function plausible. The sulphite-, thiosulphate-, and tetrathionate-reductases of *Desulphovibrio desulphuricans* perform a metabolic function analogous to the cytochrome c oxidase of aerobes; it seems probable, but is not proven, that the sulphate-reductase acts similarly. A cytochrome of the b group may perform a similar function in nitrate reduction by *Escherichia coli* (Sato & Egami, 1949) and *Pseudomonas stutzeri* (Allen & van Niel, 1952); a cytochrome of the c group is involved in the oxidation of nitrite by *Nitrobacter* spp. (Lees & Simpson, 1955) and in the reduction of nitrate by various denitrifying bacteria (Verhoeven & Takeda, 1956) and *Thiobacillus denitrificans* (Baalsrud & Baalsrud, 1954; Dr S. Elsdon, personal communication). Cytochrome c is concerned in the oxidation of sulphite to sulphate by plant mitochondria (Tager & Rautanen, 1956). The failure to obtain preparations of sulphate-reducing bacteria able to reduce sulphate in hydrogen may be due to (a) need for a second co-factor in the sulphate \rightarrow sulphite step, (b) chemical instability on the part of the sulphate reductase or (c) need for coupled reactions, perhaps yielding energy, in the primary attack on sulphate which are sensitive to CTAB.

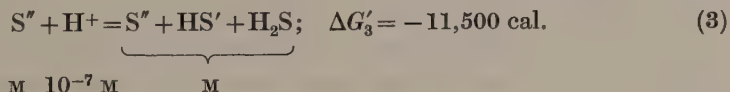
The E'_0 of cytochrome c_3 of -205 mV. is related to the free energy of its oxidation at pH 7.0. This may be written



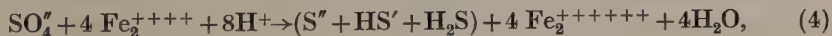
The free energy of reduction of sulphate by these bacteria may be calculated from standard free energy data (Rossini, Wagman, Evans, Levine & Jaffe, 1952)



but at pH 7.0 the sulphide ion is largely hydrolysed to $\text{HS}' + \text{H}_2\text{S}$. Using the two dissociation constants of H_2S (Hodgman, 1949) one can evaluate a correction for this:



The oxidation of reduced cytochrome c_3 by sulphate at pH 7 can be written

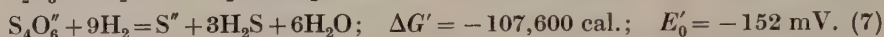
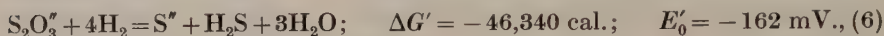
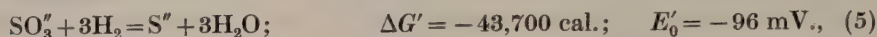


and free energy change in this reaction (ΔG_4) is given by

$$\Delta G'_4 = \Delta G'_2 + \Delta G'_3 - 4 \Delta G'_1 = -3,460 \text{ cal./mole SO}_4'' \text{ or } -865 \text{ cal./mole } c_3.$$

Thus the oxidation of cytochrome c_3 with sulphate would provide a net energy yield. The point can be expressed differently by calculating the redox potential corresponding to the reduction of sulphate in H_2 at pH 7.0 from the free energies of reactions (2) and (3), and observing that the value, $E'_0 = -188$ mV., is less negative than E'_0 of c_3 (-205 mV.).

The failure to observe complete oxidation of c_3 by sulphate and other ions, in contrast to benzyviologen, could be because sulphide was incompletely removed from solution in the test conditions, with the result that sufficient remained in solution to hold some cytochrome reduced. This explanation is unlikely, however, since the percentage oxidations quoted in Table 3 should then be independent of the substrate, which they are not. The phenomenon might be accounted for if the E'_0 values of the reductions of sulphite, thio-sulphate, etc., were of an order similar to that of c_3 ; the ions would then come to redox equilibrium with c_3 rather than oxidize it completely. If $\Delta G'$ values at pH 7 are calculated for the reductions of sulphite, thiosulphate and tetrathionate (ΔG° for $S_2O_3^{2-}$ obtained from Mel, 1954), in the manner used above for sulphate, and the values are converted to potentials, the quantities below are obtained:



These potentials do not lie in the order necessary to account for the results in Table 3; hence no simple thermodynamic formulation of the reactions involved will account for the phenomenon.

The low E'_0 values of cytochrome c_3 and of the reduction of sulphate, sulphite, etc., are consistent with the strictly anaerobic habit of these bacteria. Starkey & Wight (1945) showed that the initiation of growth of sulphate-reducing bacteria was accompanied by a decrease in redox potential of the environment to below -200 mV.; ZoBell & Rittenberg (1948) quoted an E_h value of -100 to -300 mV. as being most favourable to growth of marine strains; Stárka (1951) showed that the ripening of medicinal muds, attributed to thermophilic strains of *Desulphovibrio*, was associated with a decline of E'_0 value to between -200 and -300 mV.; Grossman & Postgate (1953*a, b*) showed that small inocula did not multiply unless the medium was supplemented with Na_2S or cysteine, which would bring the redox potential into this range. Clearly, then, one can regard *D. desulphuricans* as an organism which conducts oxidative reactions at the strongly reducing potential of about -200 mV.

The presence of c_3 in *Desulphovibrio desulphuricans* accounts, at least partly, for the iron requirement first demonstrated by Butlin, Adams & Thomas (1949). The fact that cytochrome c_3 is present in relatively large amounts (a typical content of $0.22 \mu\text{U.}$ cytochrome $c_3/\text{g.}$ dry wt. organisms, observed spectroscopically by Barer's procedure, corresponds to a c_3 content of about 3 mg./g. ; i.e. 0.3% of the air-dry bacterial mass) and is very easily extracted and purified, makes it surprising that c_3 was not observed earlier. The explanation must lie in (a) the convention of growing *D. desulphuricans* in the presence of excess ferrous salts, thus producing a spectroscopically impenetrable black mass of bacteria and FeS ; (b) the relative difficulty of obtaining and maintaining pure cultures of these bacteria; (c) the low yields obtained in even the best batch cultures; (d) the belief, supported by earlier studies among the clostridia, that looking for cytochromes in obligate anaerobes was a waste of time.

The 630 m μ -component. This pigment, though a protein, is clearly not a cytochrome in the conventional sense of the word, and the spectroscopic resemblance to cytochrome a_2 is misleading. The present work provides no clue to its metabolic function. Oxidation and reduction lead to no spectroscopic change; there is no obvious reaction with CO, KCN or NaN₃ and no compound that can be definitely classed as a haemochrome was observed, in contrast to the report of Ishimoto *et al.* (1954*b*). The name 'desulphoviridin' is proposed for this pigment. It appears to be a simple porphyro-protein since heat, acid or alkali treatment all yield the same product: the fluorescent photo-oxidizable chromophoric group. Verhoeven & Takeda (1956) obtained a blue protein which absorbed at 600–630 m μ . from *Pseudomonas aeruginosa* during the isolation of the cytochrome concerned in nitrate reduction; but Verhoeven's pigment is clearly different from desulphoviridin since it has little absorption in the region of 411 m μ ., where desulphoviridin absorbs strongly. The chromophoric group of desulphoviridin is clearly a porphyrin-like compound since it survives contact with conc. H₂SO₄; a fluorescent metallo-porphyrin such as chlorophyll, or a metallo-bile pigment such as the red fluorescent zinc derivatives, would lose their metal in these conditions. The character of the chromophore has not been established; the type of spectrum in aqueous media was reminiscent of that of a chlorin, but the high solubility in water, which prevented a neutral spectrum being obtained, is quite uncharacteristic, because the HCl numbers of chlorin lie in the region of 15–20 %. The properties so far established would not be inconsistent with a highly carboxylated chlorin structure but are insufficient to allow a definite conclusion.

The author wishes to record his indebtedness to the very large number of authorities who have discussed this work with him and made helpful suggestions. Particularly is he grateful to Professor D. D. Woods; Dr June Lascelles (who first observed cytochrome c_3); Professor Keilin and Dr Hartree; Dr N. K. Boardman; Dr J. E. Falk; Dr K. G. Paul; Dr R. Barer; Dr Lucile Smith; Dr A. G. Ogston, all of whom spared him considerable time to discuss various points. Errors and misinterpretations are, however, the author's own. He is also indebted to Dr M. Ishimoto, Dr Jacqueline Millet and Dr W. Verhoeven for giving him access to unpublished material, and to Mr P. S. S. Dawson and his colleagues in the author's laboratory for supplying large quantities of centrifuged bacteria. This paper is published by permission of the Director, Chemical Research Laboratory.

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The Transfer of Defective *Lambda* Lysogeny between Strains of *Escherichia coli*

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SUMMARY: The infective transfer of defective *lambda* lysogeny from a defective prophage to sensitive *Escherichia coli* has been observed. The agent of transfer appears to be *lambda* phage in which has been incorporated the hereditary defective element. It is concluded that the existence of the phage genes responsible for defective lysogeny is not limited to the prophage condition.

Those strains of *Escherichia coli* which are lysogenic for phage *lambda* may be induced to liberate phage by exposing the organisms to ultraviolet (u.v.) light: each induced organism subsequently liberates about 100 *lambda* phage particles (Weigle & Delbrück, 1951). There exist, however, defective *lambda* lysogenic organisms in which the yield of phage particles averages less than 10^{-5} /induced bacterium. The cause of this defect appears to be a mutation of a prophage gene, for it recombines with other prophage markers during the bacterial growth of a doubly lysogenic strain (Appleyard, 1954*b*). Unlike all other prophage genes, the one responsible for defective lysogeny has been observed to affect only the number of mature phage particles liberated after induction of bacteria carrying the defective prophage; none of the phage liberated has yet been shown to transmit the defect. As long as this is true, the genetic status of the defect remains in doubt. The 'mutation' might, for example, be no more than a change in relationship between part of an unaltered prophage and the genetic apparatus of the bacterium. Such a relationship has meaning only in the lysogenic complex, so that any mutation of this kind would be inherently restricted to the prophage condition. I have therefore designed experiments to find out whether the mutation responsible for defective lysogeny can be transmitted by extracellular *lambda* phage.

METHODS

Bacteria and bacteriophages. *Lambda* phage and some of its mutants, together with sensitive and lysogenic strains of *Escherichia coli*, were described previously (see Appleyard, 1954*a, b*). In the present paper prophage defects of independent occurrence are denoted as i_1 , i_2 , ..., etc. Thus, of the strains previously described C 60 becomes C 60 (λi_1), and the defective lysogenic strain derived from *E. coli* K12 by 2 min. u.v. irradiation becomes C33 (λi_2).

Methods. The methods used were those previously described (Appleyard, 1954*a, b*), with the additions and modifications noted below.

Magnesium and other supplements. Observations kindly communicated to me by A. D. Kaiser have led me to supplement all media, unless otherwise stated,

with 0.25 % (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in order to improve *lambda* absorption. Traces of Ca and Fe and 5 μg . thiamine hydrochloride/ml. were also added to all growth media.

Techniques involving weak virulent lambda-v 1. (1) *Cross-streak test.* The primary technical problem throughout was to detect and assay defective lysogenic organisms among the survivors of experimental *lambda* infections, which include both sensitive organisms and *lambda*-resistant mutants. In contrast to these, both defective and non-defective ('healthy') lysogens are immune to weak virulent *lambda*-v1 but sensitive to strong virulent *lambda*-v2. In cross-streak tests all four types of organism may be identified as in Table 1. This cross-streak scheme was my basic method of identification of organisms carrying defective *lambda* prophage. Colonies which resist *lambda* phage because they do not adsorb it cannot be tested for lysogeny by cross-streaking against *lambda*-v1. These were never sufficiently numerous to cause any appreciable error in my experiments.

Table 1. *Cross-streak method for the identification of defective lysogenic organisms*

Cross-streak against:	...	<i>lambda</i> - v1	<i>lambda</i> - v2	Sensitive organisms
Type of colony				
<i>Lambda</i> resistant (V_λ^r)		R	R	—
Healthy lysogenic (Lp^+)		R	S	+
Defective lysogenic (Lp^i)		R	S	—
<i>Lambda</i> sensitive (Lp^s)		S	S	—

R=resistant; continuous streak; S=sensitive; broken streak. + =lysogenic; clear area around streak; — =non-lysogenic; no clear area around streak.

(2) *Replica test.* The cross-streak method becomes impractical when sufficient survivors of infection must be examined to obtain significant quantitative results. In some early experiments I partially overcame this difficulty by destroying most of the sensitive survivors with a great excess of *lambda*-v1. Later I devised the following *lambda*-v1 replica test.

On a nutrient plate is poured a top layer consisting of 0.1 ml. of a *lambda*-v1 suspension whose titre exceeds 2×10^{11} plaque-forming particles/ml., well mixed with 3 ml. 1.5 % nutrient agar. The plate is stored at 4°. When it is desired to examine a plate on which colonies have grown from the organisms which survived an experimental *lambda* infection, test colonies are imprinted on the *lambda*-v1-seeded plate by the usual velveteen method (Lederberg & Lederberg, 1952). The plates are incubated for 4–6 hr. at 37° and examined. The replicas of sensitive colonies appear as very thin spotted growth, while those of the other three classes in Table 1 appear as normal thick growths. In this way *lambda*-sensitive organisms on the original plate are identified.

Double replica test. The method just described is generally used in conjunction with a second replication of the same original plate on to a plate seeded with sensitive bacteria; this second replica plate is then exposed before

incubation to u.v. light for 15 sec. Organisms from a healthy lysogenic colony imprinted on the plate are thereby induced to liberate phage particles which form a clear area or halo round the replica colony: these halos therefore enable healthy lysogenic colonies on the original plate to be identified. Colonies which contain neither sensitive nor healthy lysogenic organisms are provisionally classed as consisting of defective lysogenic organisms and are picked, grown in nutrient broth and retested by the full cross-streak scheme of Table 1, which also serves to eliminate truly λ -resistant organisms. I confirmed by picking and cross-streaking, that healthy lysogenic colonies could be reliably counted from the halos on the appropriate replica plates: thus only suspected defective lysogens needed to undergo the full cross-streak test. Sectoring or mixed colonies were disregarded when counting either healthy or defective lysogens, since a much smaller sector could be recognized as healthy lysogenic than as defective lysogenic. The method was found to be quantitatively accurate in tests made on artificial mixtures of defective and healthy lysogenic with sensitive organisms.

Preparations of phage suspensions. To prepare phage suspensions from doubly lysogenic and other strains, I selected bacterial derivatives resistant to λ (V_λ) so as to prevent readsorption of phage and proceeded as follows.

A saturated (overnight) culture was diluted 1/2000 in fresh broth and grown with aeration at 37° to a viable bacterial count of 3×10^8 /ml. (approx. 2 $\frac{3}{4}$ hr.). The suspension was centrifuged at 2000 *g* for 10 min., the supernatant fluid discarded and the pellet resuspended in buffer. The resuspended bacteria were exposed to u.v. for 15 sec. in a layer not more than 1 mm. thick, while the vessel was gently swirled by hand. Larger volumes (200 ml.) were occasionally irradiated in thicker layers with mechanical stirring, and a suitably adjusted dose of u.v. irradiation. To the suspension was added one-ninth its volume of a solution of 10 % tryptone. The irradiated culture was then aerated at 37°. Precautions were taken to prevent photoreactivation for the first 30 min. after induction; 120 min. after the addition of tryptone, the suspension was assayed for phage, suitably sterilized and re-assayed. Sterilization was carried out either by filtration or by shaking with sufficient chloroform to saturate the aqueous phase. In the latter case the suspension can be stored over chloroform in the cold. In agreement with Markovitch (1954) I found a slight but uniform decrease in phage titre on shaking with commercial chloroform. Suspensions which were to be compared with cyanide-treated suspensions were dialysed immediately after chloroform treatment (see below).

Cyanide + chloroform dialysis technique. I occasionally found it desirable to control the burst size in different portions of the same u.v.-induced culture. In agreement with Weigle & Delbrück (1951), I found the addition of cyanide to be satisfactory, provided that this was done not less than 50 min. after induction. Phage liberation was complete about 20 min. after the cyanide was added. I used the procedure described below.

A culture was grown and irradiated as described earlier; 45 min. later it was divided into suitable portions in separate bubbler tubes. At various times

thereafter (t), potassium cyanide was added to the individual tubes to a final concentration of 0.01 M. At time $t+30$ min. each tube was shaken with chloroform and at once pipetted into a sack formed of dialysis tubing previously sterilized by autoclaving. The neck of the sack was tied round a glass tube plugged with cotton-wool to permit sterile access. The sack was at once suspended in about 3 l. sterile ice-cold nutrient broth or buffer and allowed to dialyse for 24 hr. Dialysis was repeated against fresh liquid for a further 24 hr. and the phage stock withdrawn with a volumetric pipette. The last residue in the sack was generally discarded, as it was apt to contain precipitated material. I usually relied solely on convection and an occasional swirl by hand for the necessary stirring. No change of phage titre was observed during the dialysis procedure, nor untoward effects of cyanide or chloroform in working with suspensions prepared in this manner.

RESULTS

It is convenient to consider the transmission of defective lysogeny from one organism to another by extracellular phage in terms of three separate postulates:

(i) That sensitive bacteria can acquire defective lysogeny as a consequence of infection by a cell-free phage suspension which has been prepared through the u.v.-induction of suitable lysogenic organisms.

(ii) That only those suspensions which originate from bacteria containing a defective prophage can transmit defective lysogeny, under the conditions of (i).

(iii) That the infective agent responsible for the transfer of defective lysogeny is *lambda* phage.

The experimental evidence for each of these postulates will be described in turn.

The acquisition of defective lysogeny by sensitive bacteria

I reasoned that phage in which the genetic defect of the prophage had been incorporated was *a priori* most likely to be found in the phage suspensions prepared by u.v. induction of doubly lysogenic bacteria having one healthy and one defective prophage; such an arrangement ensures that the healthy prophage carries out all the activities related to the vegetative growth and maturation of *lambda* phage in the immediate presence of the genetic defect. In consequence, I worked extensively with cell-free phage suspensions of this kind. The results of a typical experimental infection of sensitive bacteria by such a suspension are shown in Table 2. The defective lysogenic survivors of infection were counted by the double replica method confirmed by cross-streaking against both weakly virulent ($v1$) and strongly virulent ($v2$) *lambda* phage, and it was necessary to show that this cross-streak test reliably selected defective lysogenic colonies under the conditions of our experiment. That the characteristic resistance to *lambda-v1*, but not to *lambda-v2*, is a hereditary property of the bacteria of such a colony, and not a temporary phenomenon due to a mixture of cell-types, was shown by

resuspending samples of 10 colonies and re-testing 10 subcolonies derived from each. All 100 subcolonies possessed the same characteristic resistance pattern as their parents. That the resistance pattern was a true indication of defective lysogeny was shown by examining a total of 46 such colonies drawn from several experiments (including second-generation colonies from the 10 just mentioned). From each, a broth culture was grown to the end of the logarithmic phase, induced by 15 sec. u.v. irradiation and the organisms resuspended

Table 2. *Infective acquisition of defective lysogeny by sensitive organisms from a phage suspension prepared by u.v. induction of CR 751 (λi_1 , λv_1)*

Infection tube	Input of sensitive organisms	$2.8 \times 10^8/\text{ml.}$
	Input of <i>lambda</i>	$4.2 \times 10^8/\text{ml.}$
	Adsorbed <i>lambda</i>	$3.7 \times 10^8/\text{ml.}$
	Bacteria yielding phage	$1.3 \times 10^8/\text{ml.}$
	Bacteria surviving infection	$1.2 \times 10^8/\text{ml.}$
Bacterial survivors	Defective lysogenic	$6.5 \pm 0.7 \times 10^6/\text{ml.}$
	Healthy lysogenic	$3.2 \pm 0.13 \times 10^7/\text{ml.}$
	Sensitive organisms	$7.9 \pm 0.5 \times 10^7/\text{ml.}$
	Genetically resistant organisms	0 ($< 5 \times 10^4/\text{ml.}$)
Uninfected culture	Sensitive organisms	$1.3 \times 10^9/\text{ml.}$
	Others (2490 colonies examined)	0 ($< 6 \times 10^6/\text{ml.}$)

Sensitive organisms grown to a viable count of about $10^9/\text{ml.}$ were mixed at 37° with a phage suspension in nutrient broth. 15 min. were allowed for adsorption. Bacterial survivors and organisms plated directly from the uninfected sensitive culture were classified by the double replica method confirmed by cross-streaking. \pm : standard deviation due to purely statistical errors. 0 ($< n$): one observed would have corresponded to n .

in broth. In every case the optical density of the culture fell precipitately 80–90 min. later, and after 120 min. a yield of between 5×10^{-8} and 5×10^{-5} phage particles/bacterium exposed to u.v. was present in the suspension. These are the characteristic properties which define the defective lysogeny under investigation (Appleyard, 1954*b*). Results similar to those of Table 2 were obtained by another method, in which those genetically sensitive bacteria which survived infection were destroyed through immediate exposure of all the survivors to a high multiplicity of weak virulent *lambda*.

In the course of 40–50 experiments, by both methods, in which infection of sensitive bacteria was followed by the recovery of defective lysogenic organisms, I did not observe even one defective lysogenic bacterium in the uninfected sensitive cultures. I conclude that sensitive bacteria can acquire defective lysogeny from certain phage suspensions.

The transfer of defective lysogeny

It remains possible that, in the experiments I have described, defective lysogeny arose by some form of aberrant infection rather than by transfer. To eliminate this possibility, it was necessary to show that a phage suspension could only confer defective lysogeny upon sensitive cells if it originated from bacteria which carried a defective prophage. Each such suspension was therefore compared with an otherwise identical suspension prepared from organisms

which contained only healthy prophage. I first superinfected two singly lysogenic strains of *Escherichia coli*, one carrying a defective, the other a healthy but otherwise identical prophage, with the same suspension of the phage mutant *lambda-cl* and so prepared a pair of doubly lysogenic bacterial strains identical in bacterial and phage genotype, except that one of them carried a defect in one prophage. Phage suspensions were made from both strains by u.v. induction and assayed for their ability to confer defective lysogeny. As the measure of this ability I used the ratio of defective to healthy lysogenics (Lp^+/Lp), which they formed upon infection of identical sensitive organisms.

The results of such an experiment are shown in Table 3, of which the last column shows that the ability to confer defective lysogeny was restricted to the phage suspensions prepared by u.v. induction of the strain C 112 ($\lambda i_1, \lambda cl$) containing a defective prophage. In principle, such a restriction might appear to exist if, when a suspension of phage was prepared, its ability to confer defective lysogeny were very strongly correlated with the average yield of healthy phage particles per induced bacterium, that is, with the burst size. To eliminate this possibility three phage suspensions were made and compared (Table 3) the burst sizes being: (1) low (2.8); (2) high (29); (3) intermediate (15). The necessary low average yields were obtained by the cyanide procedure mentioned earlier.

Table 3. *The ability of phage suspensions to confer defective lysogeny upon sensitive organisms, and its correlation with descent from a defective prophage*

Origin of phage suspension		Bacterial survivors of test infection examined	Defective lysogenics (Lp^+)	Healthy lysogenics (Lp^-)	$Lp^+/Lp^- \times 100$
From defective prophage, by u.v. induction of C 112 ($\lambda i_1, \lambda cl$)	(1)	2500	16	234	6.8 ± 1.8
	(2)	8000	18	181	9.9 ± 2.4
From healthy prophage only, by u.v. induction of C 112 ($\lambda +, \lambda cl$)	(3)	3200	0	248	0 (< 0.4)

Each suspension infected part of the same culture of sensitive organisms (C 600) and was assayed as in Table 2. \pm : standard deviation due to purely statistical errors; 0 ($< n$): one observed would have corresponded to n .

Any comparison of the kind just described includes, as well as a doubly lysogenic strain with a defect in one of its prophages, a similar strain without the defect. I therefore wished in each case to demonstrate the presence or absence of the lysogenic defect by a method independent of the origin of the doubly lysogenic strain. To do this, I used the tendency previously reported (Appleyard, 1954*b*) for the prophage characters of doubly lysogenic strains to segregate during bacterial growth.

Portions of fully grown cultures were suitably diluted and spread upon nutrient plates. The colonies which grew up were tested for lysogeny by replica

plating, the predominant type giving rise to mottled halos. Those colonies which arose from bacterial segregants that had lost one or more prophage characters were counted according to the following scheme:

(i) Clear halo (*cl*): colony has lost the prophage character for turbid plaque formation and retained only that for clear plaque formation.

(ii) Turbid halo (*t*): colony has lost the prophage character for clear plaque formation and retained only that for turbid plaque formation.

(iii) No halo (*i*): colony has lost all healthy and contains only defective prophages.

The ratio of the third class of segregants to the sum of the first two, $i/(t+cl)$, was considered to provide a quantitative test for the presence or absence of defective prophage, whatever the overall frequency of segregations. The application of the method to the bacterial strains mentioned in Table 3 is shown in Table 4.

Table 4. Segregation of defect in doubly lysogenic strains

Strain	Segregants observed		
	Clear or turbid halo (<i>t+cl</i>)	No halo (<i>i</i>)	$i/(t+cl)$
C112 ($\lambda i_1, \lambda cl$)	35	51	1.45
C112 ($\lambda +, \lambda cl$)	202	0	0 (<0.005)

The halos were observed around replica colonies on plates seeded with sensitive bacteria. The plates were exposed to u.v. for 15 sec. after replication but before incubation. 0 (<*n*): one observed would have corresponded to *n*.

The restriction demonstrated in Tables 3 and 4 still holds when the lysogenic defect i_2 is employed instead of i_1 . It is unaffected by the bacterial genotype of either the strains from which the phage suspensions are prepared or the sensitive bacteria upon which defective lysogeny is conferred: either culture can be F⁺, F⁻, prototrophic or multiply auxotrophic (leucine, threonine, thiamine dependent, or cystine, histidine dependent).

The fact that a phage suspension can confer defective lysogeny upon *lambda*-sensitive bacteria when and only when it is descended from defective prophage implies that the defect in the newly formed lysogenic bacteria does not, under the conditions of my experiments, arise *de novo* at infection, but is in each case transferred from the original defective prophage by some agent in the phage-containing suspension. The bacterial strain C112 ($\lambda i_1, \lambda cl$) of Table 3 originally obtained its defective prophage by infection. The experiment described in Table 3 is therefore an example of serial transfer of defective lysogeny.

Identification of the agent of transfer with lambda phage

I compared three properties of the carrier of the lysogenic defect in suspensions which contain it with those of the plaque-forming phage present in the same suspensions. In each case the suspension was partitioned or treated so as to modify grossly its content of *lambda* plaque-forming particles.

The ratio of defective to healthy lysogenic organisms formed by the suspension among sensitive bacteria was measured before and after treatment. Tables 5-7 show that within the limits of experimental error the carrier of defective lysogeny had the same mass as *lambda* phage, the same rate of inactivation by specific anti-*lambda* serum, and the same rate of absorption by bacteria which absorbed *lambda* (V_{λ}^s organisms). I regard these three properties as sufficient to identify the agent of transfer of defective lysogeny with *lambda* phage.

Table 5. *Centrifugal test of identity of carrier of defective lambda lysogeny with lambda phage*

Infesting phage	Ratio of defective to healthy lysogenics formed in sensitive organisms: $Lp^i/Lp^+ \times 100$
Original suspension (prepared from u.v. induced CR 751 ($\lambda i_1, \lambda v1$))	13 ± 3.0
Precipitate (c. 80 % of phage)	9.4 ± 2.3
Supernatant (c. 10 % of phage)	12 ± 2.7

10 ml. of the suspension were spun for 2 hr. in the cold at 25,000 g (sufficient to precipitate 80 % of the phage). The top 8 ml. of supernatant were withdrawn and the pellet was resuspended in the remaining 2 ml. The original stock, resuspended pellet and supernatant were assayed by the double replica method as in Table 2. \pm : standard deviation of result due to purely statistical errors.

Table 6. *Immunological test of identity of carrier of defective lambda lysogeny with lambda phage*

Infesting phage	Percentage of plaque-formers surviving treatment	Ratio of defective to healthy lysogenic organisms formed from sensitive cells: $Lp^i/Lp^+ \times 100$
Untreated suspension (prepared from u.v. induced CR 751 ($\lambda i_1, \lambda v1$))	100	8.7 ± 1.2
Suspension exposed to anti- <i>lambda</i> serum at 48°	5.3	6.3 ± 2.1
Suspension exposed to control rabbit serum at 48°	64	10.3 ± 2.5

Separate portions of the suspension were exposed for 4 hr. at 48° to anti-*lambda* rabbit serum previously absorbed by bacteria, and to control rabbit serum. Fractions of these and of the original suspension were assayed by the double replica method as in Table 2. \pm : standard deviation of result due to purely statistical errors.

The carrier of defective lysogeny is not, however, a mere appendage or incorporated part which confers on normal *lambda* a low probability of defective lysogenization. When cultures of defective lysogenic organisms were induced by exposure to u.v. light the resulting suspensions contained a few particles able to confer defective lysogeny upon sensitive bacteria. Although these defective phage appeared to be much fewer in number than the bacteria which liberated them, they outnumbered the plaque-forming *lambda* phage in the same suspensions by a factor of 100 or more. For example, a suspension prepared by the u.v. induction of C 33 (λi_2) contained 1.9×10^3 /ml. plaque-

forming *lambda* particles, but was able to form 2×10^5 /ml. defective lysogenic organisms upon infection of the sensitive strain C112.

We conclude that the carrier of defective lysogeny is defective *lambda* phage, in the special sense that it carries the genetic defect responsible for defective lysogeny.

Table 7. *Specific adsorptive test of identity of carrier of defective lambda lysogeny with lambda phage*

Infesting phage	Percentage of plaque-formers surviving treatment	Ratio of defective to healthy lysogenics formed in sensitive organisms: $Lp^i/Lp^+ \times 100$
Untreated suspension (prepared from u.v. induced Cr 751 ($\lambda i_1, \lambda \nabla 1$))	100	9.5 ± 2.2
Suspension exposed to λ -sensitive organisms	33	12.0 ± 3.5
Suspension exposed to λ -resistant (V_{λ}^r) organisms	100	18.0 ± 4.0

Separate portions of the suspension were exposed for 15 min. at 37° to bacteria at a concentration of $2 \cdot 10^8$ /ml. In one case the bacteria were *lambda*-sensitive (C 600); in the other *lambda*-resistant (C 600/ λ). The bacteria were removed by 5 min. centrifugation at 7000 *g* at 4° , and each sample was sterilized with chloroform. Portions of each sample and of the original suspension were assayed by the double replica method as in Table 2. \pm : standard deviation of result due to purely statistical errors.

DISCUSSION

Defective lysogeny of a kind similar to that investigated here was first described by Lwoff & Siminovitch (1951) in a strain of *Bacillus megaterium*. Biochemical evidence led Siminovitch (1951) to conclude that the defect in their

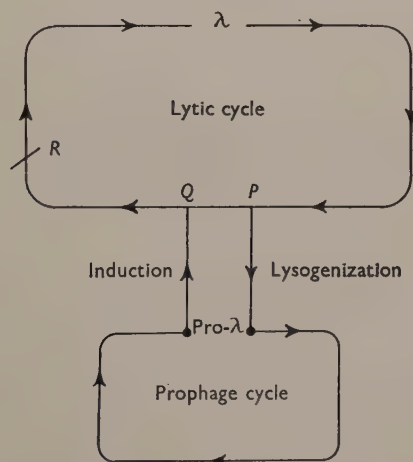


Fig. 1. The location of the block in the life-cycle of defective *lambda* phage.

strain brought about a failure to resume synthesis of deoxyribonucleic acid after u.v. induction. The existence of defective lysogeny was recognized in *Escherichia coli* by Lederberg & Lederberg (1953). I have previously

concluded (Appleyard, 1954*b*) that the defect in the lysogeny is caused by a mutation of a prophage gene.

The present experiments show that the mutant characters concerned in two such defects can be incorporated into extracellular *lambda* phage. The prophage genes controlling these defects therefore, like other known prophage genes (Appleyard, 1954*b*), appear to be transmissible to mature phage: their existence is not limited to the prophage state. Because defective phage particles form no plaques, it is apparent that each defect constitutes a block in the life-cycle of the phage at some point during the lytic cycle beyond *Q*, in Fig. 1. Such a hypothetical block has been inserted at *R* in that figure.

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The Chemical Composition of the Cell Wall in some Gram-positive Bacteria and its Possible Value as a Taxonomic Character

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SUMMARY: Hydrolysates of cell-wall preparations of more than 60 strains of corynebacteria, lactobacilli, streptococci, staphylococci and other Gram-positive cocci have been examined by paper chromatography. A very high proportion of the amino acid moiety of the cell-wall complex could in each case be accounted for in terms of 3 or 4 of the amino acids alanine, glutamic acid, lysine, diaminopimelic acid, aspartic acid and glycine. These were associated with varying combinations of sugars and amino sugars. In general, each bacterial genus appears to have a characteristic pattern of cell-wall components, particularly in regard to the amino acids present. Variations in the relative proportions of the sugars appear to differentiate the individual species within a genus. The possible value of cell-wall composition as a taxonomic character is discussed.

It is now well established that the insoluble fraction obtained by centrifuging suspensions of bacteria which have been mechanically disintegrated is largely composed of cell-wall fragments, and a number of workers have reported on chemical analyses of such fractions from several bacterial species (Mitchell & Moyle, 1951; Holdsworth, 1952; Salton, 1952, 1953). The present investigation was undertaken when it was found that the cell-wall compositions of several species of corynebacteria resembled each other closely, but differed markedly from those of streptococci. This finding, coupled with the anomalous results obtained with a strain of *Corynebacterium pyogenes*, suggested that a more systematic investigation of species from different bacterial genera might be profitable. The present paper deals with the results obtained so far in a survey of corynebacteria, lactobacilli, streptococci, staphylococci and a number of other Gram-positive cocci.

The general method has been to grow the organisms on a suitable medium, disintegrate the washed suspension by shaking with ballotini in a Mickle disintegrator, and purify the cell-wall (insoluble) fraction by treatment with proteolytic enzymes. These purified preparations were then hydrolysed in acid and the hydrolysates examined by paper chromatography. Some of the results have been briefly reported previously (Cummins & Harris, 1955).

MATERIALS AND METHODS

Strains of bacteria

Strains obtained from the National Collection of Type Culture (NCTC) or from the National Collection of Industrial Bacteria (NCIB) are listed as such, with their numbers. The sources of other strains were as follows:

Corynebacterium diphtheriae (gravis, mitis and intermedius) from Dr Donald Payne, Public Health Laboratory, Northallerton; *C. ulcerans* (gelatin-liquefying, starch-fermenting) from Dr W. H. H. Jebb, Public Health Laboratory, Oxford; *C. hofmanni*, isolated in the Clinical Laboratory, London Hospital, from throat swabs; *C. xerosis*, isolated in the Clinical Laboratory, London Hospital, from conjunctival swabs; *C. renale*, obtained from Dr F. C. O. Valentine, London Hospital (originally from Professor R. Lovell, Royal Veterinary College); *C. pyogenes*, strains Wye 1, 2, 3 from Veterinary Investigation Centre, Wye, Kent (Mr J. D. Paterson); strains 637 and 13081 from Dr Lane Barksdale, N.Y. University. *C. haemolyticum* strain 53/W/1 was also obtained from Dr Barksdale, and represents the type strain (MacLean, Liebow & Rosenberg, 1946).

Streptococcus pyogenes (group A). Isolated in the Clinical Laboratory, London Hospital, from a throat swab. Streptococci of other Lancefield's groups were obtained from the National Collection of Type Cultures. *Staphylococcus aureus*, strains 1, 2 and 3, isolated from nasal swabs, all coagulase-positive; *S. albus* strains 1, 2 and 3, isolated from nasal swabs, all coagulase-negative. These strains were picked as 'aureus' or 'albus' on colonial pigmentation, and were later tested for coagulase production. *S. citreus*: selected from culture of nasal swab as giving typical lemon yellow pigment, coagulase-negative.

Lactobacilli spp. The strain originally examined was obtained from a sample of yoghurt, and purified by plating out on tomato juice agar. It has not been further identified. Subsequent strains were obtained from the National Collection of Industrial Bacteria.

Identification and naming of strains

In most cases strains of known origin have been used, either from the NCTC or NCIB. These were checked for purity, but not investigated further. Strains of *Corynebacterium diphtheriae* had been isolated from cases, and had the typical morphology and fermentation reactions. Strains of *C. hofmanni* isolated from throat swabs were accepted as such if they stained evenly with methylene blue except for a central unstained bar, were strongly Gram-positive and failed to ferment glucose, maltose and sucrose. The strains designated *C. xerosis* were heavily barred diphtheroids, isolated from conjunctival swabs, which fermented glucose and sucrose and gave rather small dry rough colonies. The single strain of *C. murium* was a non-acid fast corynebacterium, isolated from caseating lesions in a mouse.

With two exceptions strains are listed under the names which they bore when received, the exceptions being two strains of lactobacilli. These were originally received as *Lactobacillus bulgaricus* (NCTC 76) and *L. bifidus* (NCTC 2797), but we were informed by Dr Dorothy Wheeler that according to physiological and serological tests (Wheeler, 1955*a, b*; Sharpe, 1955; Briggs, 1953) these should be reclassified as *L. helveticus* (76) and *L. fermenti* (2797) respectively. With the agreement of the Curator of the National Collection of Type Cultures, Dr Cowan, we have therefore altered the names but retained the NCTC numbers.

Culture media

Streptococci, aerococci and strains of *Corynebacterium pyogenes* were grown on infusion broth to which had been added before sterilization 0.1 % (w/v) sodium phosphate (Na_2HPO_4). To this broth was added before inoculation a sterile glucose + bicarbonate solution (10 % glucose, 10 % NaHCO_3) in the proportion of 2 ml. to 100 ml. broth. In the case of *C. pyogenes* growth was improved by the further addition of 2 % sterile horse serum.

Staphylococci were grown on nutrient agar in Petri dishes, or in 10 × 8 in. metal trays with metal lids; but some more slowly growing strains such as *Micrococcus conglomeratus* were grown in nutrient broth at 28° in conical flasks to give good aeration.

Lactobacilli were grown in tomato juice broth (Briggs, 1953) in large screw-capped bottles containing 1 l., for 48 hr. at 37°. The suspensions so obtained were usually dirty brown in colour, even after washing, but the cell-wall preparations from them were pure white, as with other organisms.

Preparation of cell-wall suspensions

Bacteria were harvested from solid media by washing off in distilled water, or collected from liquid media by centrifugation. Pathogenic organisms were killed by heating at 60° for 1 hr. or, in the case of relatively heat-resistant species, with formalin (0.5 %) overnight at room temperature. These precautions were omitted in the case of non-pathogens.

Suspensions were washed twice in distilled water and the bacteria disintegrated with ballotini (Chance no. 12) in a Mickle tissue disintegrator, using 4 g. ballotini and 6 ml. bacterial suspension in each cup. Shaking was done at 4°, and the process judged complete when no intact organisms could be seen in smears stained by Gram's method. The rubber stoppers of the cups were protected by cellophane as suggested by Hotchin, Dawson & Elford (1952).

After shaking, the cups were centrifuged at 1000 r.p.m. for 5–10 min. to break the froth, and the supernatant decanted. The ballotini were washed three times, with a few ml. of distilled water each time, and the washings added to the original supernatant. This was centrifuged at low speed (c. 1000 r.p.m.) for a short time to remove glass beads, and then at 3000–4000 r.p.m. until the crude cell-wall fraction was deposited. This was washed once in distilled water, resuspended in 0.05 M-phosphate buffer (pH 7.6) and digested with crystalline trypsin and ribonuclease (Armour) at 37°. Both enzymes were used at 0.5 mg./ml. Digestion was continued for 2–3 hr. and a considerable decrease in opacity generally occurred during this period. The mixture was then centrifuged, the deposit washed twice in distilled water, resuspended in 0.02 N-HCl with 1 mg. crystalline pepsin (Armour)/ml. and digested at 37° for 18–24 hr. After peptic digestion, the material was finally washed several times in distilled water.

If not hydrolysed immediately, the cell-wall preparations were preserved as

suspensions in distilled water + 0.3 % sodium azide. Such preserved material was centrifuged and washed once in distilled water before hydrolysis.

Ideally, each sample should have been checked by electron microscopy before hydrolysis, but this was impossible. However, a few samples chosen at random were so examined, and these showed that the method gave adequately pure material.

Hydrolysis

The amount of material used for hydrolysis depended to some extent on the size of the sample available, but in general about 50 mg. (dry weight) was used, divided so that two-thirds was used for the investigation of sugars, and the remaining one-third for amino acids.

For sugars. Samples were hydrolysed in 2 N-H₂SO₄ in sealed tubes in a water bath at 100° for 2 hr. After cooling, the hydrolysates were filtered, neutralized with solid Ba(OH)₂ and centrifuged, and the supernatant evaporated to dryness *in vacuo* over P₂O₅. The final product was redissolved in 0.2–0.25 ml. distilled water.

For amino acids and hexosamines. Samples were hydrolysed in 6 N-HCl in sealed tubes at 100° for 8 hr., or in some cases at 105° for 18–24 hr. They were then filtered, evaporated to dryness on a boiling water bath, and finally redissolved in 0.2–0.25 ml. distilled water.

Various degrees of humin formation were encountered with cell-wall preparations from different organisms. It was most marked in the case of corynebacteria. The humin was filtered off before evaporation on the water bath.

Chromatography

Amino acids and amino sugars. In all cases two-dimensional descending chromatograms (22 × 18½ in. Whatman no. 4 filter-paper) were prepared. Phenol+water (80 : 20) in an ammoniacal atmosphere were used for the first solvent, and lutidine+water (65 : 35) for the second. The amount of cell-wall hydrolysate chromatographed in each instance corresponded to about 5 mg. dry weight of whole organisms. The spots were revealed by dipping the chromatogram rapidly through a solution of 0.2 % ninhydrin in 95 % acetone and 5 % water, and after it had dried heating at 105° for 5 min. In general, the amino acids present could be readily identified from the position and colour of the spots obtained. Where there was any uncertainty appropriate markers were added to the hydrolysate. Further confirmation of the identity of the spots was also obtained in certain instances by ionophoresis in buffers at different pH values. The Elson and Morgan reaction and the reaction with ammoniacal silver nitrate (Partridge & Westall, 1948) were also used to differentiate the amino sugars from the amino acids and to confirm the identification of glucosamine and galactosamine.

In most cases after 8 hr. hydrolysis in 6 N-HCl the only ninhydrin reacting spots present corresponded to known amino acids and amino sugars, and the hydrolysis was therefore regarded as complete. There were, however, two exceptions to this. First, in cell-wall preparations from all the lactobacilli

examined. With the exception of the various strains of *Lactobacillus plantarum*, hydrolysis in 6 N-HCl for 8 hr. revealed strong spots corresponding to aspartic acid, glutamic acid, alanine and lysine, and also a strong unknown spot with R_f values about the same as lysine in phenol + NH_3 and as glycine in lutidine. On further hydrolysis up to 24 hr. this spot gradually became weaker till it disappeared almost entirely. *Pari passu* there occurred a progressive intensification of the spots corresponding to aspartic acid and lysine, and possibly a less pronounced increase in the glutamic acid and alanine spots. The unknown material was therefore regarded as a peptide relatively resistant to hydrolysis, and probably composed largely of aspartic acid and lysine. No further investigation of this point has yet been undertaken, but for the purposes of the present paper, hydrolysis of the cell-wall preparations in this group of organisms was regarded as complete after 24 hr. in 6 N-HCl at 105° . Secondly, in all the cell-wall preparations examined there was found a ninhydrin reacting substance, moving in phenol + NH_3 somewhat more slowly than either glucosamine or galactosamine, and in lutidine at about the same rate as these amino sugars. In lutidine, and to a lesser extent in phenol + NH_3 , its R_f value was somewhat variable from run to run. The colour after development with ninhydrin was much the same as that given by glucosamine, and it was found to react with the Elson and Morgan reagents and with ammonical silver nitrate in the same way as glucosamine and galactosamine. It did not correspond in chromatographic behaviour or in reactions to any known naturally occurring amino acid or amino sugar. Prolongation of the hydrolysis up to 24 hr. indicated that it was about as stable as glucosamine and galactosamine under these conditions, and it did not appear to be disrupted into any additional ninhydrin-reacting substances. This material seems to correspond closely in properties to the unidentified hexosamine reported by Strange & Powell (1954) in a soluble peptide obtained from cultures of germinating spores of *Bacillus subtilis*, *B. cereus* and *B. megaterium*. In the rest of this paper the material will be referred to as 'unknown "hexosamine"'.

Sugars. Adequate resolution of the complex mixtures of sugars encountered in many of these cell-wall hydrolysates could not be obtained by uni-dimensional chromatography. As a routine, therefore, two-dimensional chromatograms ($22 \times 18\frac{1}{2}$ in., no. 4 Whatman filter-paper) were prepared using phenol + water as the first solvent and lutidine + water as the second. The spots were revealed by dipping in a reagent mixture containing: aniline 2.0 ml., phthalic acid 3.3 g., acetone 95 ml., and water 5 ml.; followed by heating at 105° for 5–10 min. In general, an amount of hydrolysate corresponding to about 20 mg. dry weight of whole bacteria was used in the preparation of each chromatogram.

The distribution of the sugars on the chromatograms was substantially as described by Partridge & Westall (1948), who used phenol + NH_3 and collidine as their solvents. In our experiments ammonia was omitted from the phenol run because it was found to lead to excessive streaking of the hexose and pentose spots. The only disadvantage of performing the phenol run in neutral and not alkaline conditions was that the amino sugars were poorly resolved. In

comparison to the other sugars, however, these substances give a very feeble reaction with aniline hydrogen phthalate, and we relied for their identification on the methods described in the previous section. The individual hexoses and pentoses could be readily identified by their relative positions on the paper and their characteristic colour reactions with aniline hydrogen phthalate.

No attempt at accurate quantitative evaluation of the relative proportions of either the amino acids or sugars has been made. The relative amounts of the different substances present in each case have been arbitrarily graded as + + +, + +, +, \pm or trace, according to the relative sizes and intensities of the spots obtained.

RESULTS

For ease of presentation the results have been collected into several tables, each dealing with strains or species which form a related group, even though in some cases their classification is still uncertain. To bring out more clearly the pattern of amino acids, only the major components have been recorded, and the other columns left blank. The only exception to this is in the comparison between *Staphylococcus aureus* and *S. albus*, where the difference in the amount of serine appears to be significant, although it was present in amounts considerably smaller than were the other four amino acids.

Although dealt with more fully later, two points may be made at the outset. First, that the pattern of amino acid components appears to distinguish larger groups such as genera, while the species within these groups seem to be distinguished by the sugars and amino sugars which their cell walls contain. This is, however, only a broad distinction, and certain genera such as *Streptococcus* and *Corynebacterium* have distinguishing cell-wall sugars which are present in all members of the genus. Secondly, attention may be drawn to the distribution of the unusual amino acid α , ϵ -diaminopimelic acid (D.A.P.) originally described by Work (see Work, 1951) in *C. diphtheriae*. The present results confirm the suggestion of Work & Dewey (1953) that the presence or absence of this substance may be of taxonomic importance.

Streptococci

The results detailed in Table 1 show that the 8 strains examined form a homogeneous group, as far as cell-wall composition is concerned, and the distinguishing components appear to be the methylpentose, rhamnose, and the amino acids alanine, glutamic acid and lysine. A point of considerable interest is the difference in cell-wall composition between the two group D strains, a difference which is of approximately the same degree as that between either of them and, for example, the strain of group F. Salton (1953) has also examined the cell walls of strain 6782 (group D), and his results agree closely with the present findings, except that he did not detect mannose in hydrolysates of his preparation. Both Salton, and also McCarty (1952*a*, *b*) found that the polysaccharide part of the cell wall of *Streptococcus pyogenes* (group A) was

Table 1. Cell-wall composition in streptococci of different Lancefield groups

<i>Streptococcus</i> sp. Group A (1)* Group B NCTC 6175 Group C NCTC 4540 Group D NCTC 6782 Group E NCTC 5385 Group F NCTC 5389 Group G NCTC 4549	Glycine
	Serine
	Diaminopimelic acid
	Lysine	++++++
	Glutamic acid	++++++
	Alanine	++++++
	Aspartic acid
	Unknown 'Hexosamine'	++++++
	Galactosamine	+ + + +
	Glucosamine	+ + + + + +
	Mannose	+ + Tr.
	Glucose	Tr. + + + + +
	Galactose	+ + + + +
	Rhamnose	++++++
	Arabinose	

* Numbers in parentheses in this and subsequent tables indicate number of strains examined.

Table 2. Cell-wall composition in corynebacteria

<i>Corynebacterium diphtheriae</i> (4) <i>C. ulcerans</i> (3) <i>C. ovis</i> NCTC 3450 <i>C. aerosis</i> (2) <i>C. hofmanni</i> (2) <i>C. renale</i> (1) <i>C. equi</i> NCTC 1621 <i>C. murium</i> (1) <i>C. pyogenes</i> NCTC 5224 <i>C. pyogenes</i> Wye 1 <i>C. pyogenes</i> Wye 2 <i>C. pyogenes</i> Wye 3 <i>C. pyogenes</i> 13081 <i>C. pyogenes</i> 637 <i>C. haemolyticum</i> 53/W/1	Glycine
	Serine
	Diaminopimelic acid	++++++ ++++++
	Lysine ++++++ ++++++
	Glutamic acid	++++++ ++++++ ++++++
	Alanine	++++++ ++++++ ++++++
	Aspartic acid
	Unknown 'Hexosamine'	++++++
	Galactosamine	+ + + + + +
	Glucosamine	++++++
	Mannose	+ + + + Tr. + Tr. + + Tr. + + + + Tr.
	Glucose	+ + + + Tr. + + + + +
	Galactose	++++++ ++++++ ++++++
	Rhamnose	+ + + + + +
	Arabinose	++++++ ++++++ ++++++

made up of rhamnose and glucosamine, and Salton noted that D.A.P. was absent from the strain of *S. pyogenes* he examined, but that alanine, glutamic acid and lysine were present in larger amounts than other amino acids.

Corynebacteria

The first eight species in Table 2 (representing fifteen strains) make up a group whose characteristic cell-wall sugars appear to be arabinose and galactose. Although rhamnose was present in the strain of *Corynebacterium murium* it does not seem to have any more significance in this case than the presence of an approximately equal amount of mannose, when the pattern of components as a whole is considered. The distinguishing amino acids of these eight species are alanine, glutamic acid and D.A.P., which were the major amino acid components in all of them. Diaminopimelic acid has already been identified in hydrolysates of whole *C. diphtheriae* by Work (1951), and Holdsworth (1952) has shown that almost the whole of it is present in the cell-wall fraction of this species. From the same fraction, Holdsworth also obtained an oligosaccharide containing arabinose, galactose and mannose in the ratio 3 : 2 : 1, and the results given for *C. diphtheriae* in Table 2, although only roughly quantitative, agree well with these proportions. The four strains of *C. diphtheriae* examined included two mitis, one intermedius and one gravis strain, but no difference in cell-wall composition was detected between the different cultural types.

The cell-wall compositions of *Corynebacterium pyogenes* and *C. haemolyticum* (Table 2) are obviously similar to one another, but differ both in sugar and amino acid composition from the other corynebacteria since they contain neither arabinose nor galactose, and lysine appears as a major component, while D.A.P. is absent. On the other hand, rhamnose was present in both cases, and this, together with the fact that alanine, glutamic acid and lysine were the major amino acid components, suggests very strongly that these organisms are related to the streptococci in view of the results already detailed in Table 1. Six strains of *C. pyogenes* have been examined, and the results are included in Table 2. Taking into account the rather arbitrary method of estimating the amounts of constituents, these six strains showed surprisingly little variation in cell-wall composition, except for the apparently complete absence of mannose in strain 13081. However, this sugar was present only in traces in the cell walls of other strains, and the difference seems hardly significant.

Staphylococci, aerococci, sarcina and micrococci

The results in Table 3 represent the cell-wall compositions in a number of catalase-positive species of Gram-positive cocci, and several points of interest are evident. First, there is a group in which the major amino acids of the cell wall are alanine, glutamic acid, lysine and glycine, but in which no distinctive sugar appears. This group is represented by the cultures named *Staphylococcus aureus*, *S. albus*, *S. citreus*, *Sarcina lutea* and *Micrococcus luteus* (9 strains in all), and within it there seems to be a division between the strains of *Staphylococcus*

Table 3. Cell-wall composition in staphylococci, aerococci, sarcina and micrococci

Glycine	+	+	+	+	+
	+	+	+	+	+
	+	+	+	+	+
Serine	Tr.	+
Diaminopimelic acid	+	+
											+	+
Lysine	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	.	.
	+	+	+	+	+	+	+	+	+	+	.	.
Glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+
Alanine	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+
Aspartic acid
Unknown 'Hexosamine'	+	+	+	+	+	+	+	+	+	+	+	+
Galactosamine						Tr.	+	+	+	+	+	+
Glucosamine	+	+	+	+	+	+	+	+	+	+	+	+
Mannose			+				+			Tr.	Tr.	Tr.
Glucose			+	+	+	+	+	+	+	+		
			+	+	+	+	+	+	+	+		
Galactose						+	+			+	+	+
						+	+	+	+	+	+	+
Rhamnose												
Arabinose											+	+
											+	+
											+	+

Staphylococcus aureus
 (coagulase +) (3)
S. albus (coagulase -) (3)
S. citreus (1)
Sarcina lutea NCTC 611
Micrococcus luteus NCTC 8512
 (ATCC 398)
M. conglomeratus NCTC 2677
Aerococcus viridans NCTC 8251
A. viridans NCTC 7593
A. viridans NCTC 7592
A. viridans NCTC 7764
Micrococcus cinnabareus
 NCTC 7502
M. rhodochrous NCTC 7510

aureus and *S. albus*, which have a moderate amount of serine but no hexoses or pentoses, and the other three strains which contain no serine but have one or more hexoses in the cell wall. There may also be a significant difference between *aureus* and *albus* strains in respect of the amount of serine present. Both of these tentative subdivisions require confirmation by the examination of a far larger number of strains.

The second group of strains which can be distinguished among those whose cell-wall compositions are set out in Table 3 are the four strains of *Aerococcus* and probably also the strain labelled *Micrococcus conglomeratus*. Williams, Hirsch & Cowan (1953), in defining *Aerococcus* as a new bacterial genus, described it as being intermediate in many ways between *Staphylococcus* and *Streptococcus*. This is borne out by the cell-wall composition of these four strains, the essential pattern of which differs from *Streptococcus* only in that rhamnose is absent, and from *Staphylococcus* only in not containing glycine.

Thirdly, there are the two strains labelled *Micrococcus rhodochrous* and *M. cinnabareus*. These had been rejected on morphological grounds by Shaw, Stitt & Cowan (1951) and Cowan (personal communication) from their collection of Gram-positive, catalase-positive cocci, and this has been confirmed by us, since both cultures show diphtheroid forms several μ . long, particularly in the case of *M. cinnabareus*. It is interesting to see that by cell-wall composition these two strains would clearly fall into the genus *Corynebacterium* (Table 2), and the results in each case are so alike as to suggest that they may be two strains of the same species.

Lactobacilli

The results of cell-wall analyses of 7 strains (the first 7 in Table 4) show that this genus appears to be characterized by aspartic acid, alanine, glutamic acid and lysine as the major amino acids of the cell wall, but has no distinguishing hexose or pentose. The 2 strains of *Lactobacillus casei* and *L. delbrueckii* have a rather more complicated cell-wall structure than the others; galactosamine as well as glucosamine is present, both strains contain rhamnose, and the strain *L. casei* has also a small amount of arabinose. However, their amino acid pattern is the same as the other 5 strains under discussion, and all 7 seem to form a homogeneous group with the characters mentioned above.

The 4 strains of *Lactobacillus plantarum*, on the other hand, differ from the other strains of lactobacilli in three ways so far as the amino acid pattern in their cell walls is concerned: they lack aspartic acid and lysine and contain D.A.P. Diaminopimelic acid was found by Work & Dewey (1953) in the strain of *L. plantarum* which they examined, although they did not identify it as a cell-wall component since they examined hydrolysates of whole organisms.

DISCUSSION

General nature of the cell wall

The present series of results represents the analyses of cell-wall composition in nearly 60 strains, and is large enough to enable some general conclusions to be drawn as to the nature of the cell wall in Gram-positive bacteria.

Table 4. Cell-wall composition in lactobacilli

Glycine
Serine
Diaminopimelic acid	+	+	+
							+	+	+
							+	+	+
Lysine	+	+	+	+	+	+	.	.	.
	+	+	+	+	+	+	.	.	.
	+	+	+	+	+	+			
Glutamic acid	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+
Alanine	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+
Aspartic acid	+	+	+	+	+	+	.	.	.
	+	+	+	+	+	+	.	.	.
	+	+	+	+	+	+			
Unknown 'Hexosamine'	+	+	+	+	+	+	+	+	+
Galactosamine					+	+			
Glucosamine	+	+	+	+	+	+	+	+	+
Mannose	+			+	+	+		+	
				+	+	+		+	
Glucose	+	+	+	+	+	+	+	+	+
		+	+	+	+	+	+	+	+
Galactose	+			+	+	+			+
				+	+	+			+
Rhamnose					+	+			
					+	+			
Arabinose					+	+			
					+	+			

Lactobacillus sp. from Yoghurt

(unidentified)
L. acidophilus NCTC 1899

L. acidophilus, NCTC 1899

L. helveticus NCTC 76

L. fermenti NCTC 2797

L. brevis NCIB 8038

L. casei NCIB 8019

L. delbrueckii NCIB 7473

L. plantarum NCIB 8030

L. plantarum NCIB 8216

L. plantarum NCIB 7220

L. plantarum NCIB 1220
L. plantarum NCIB 8016

Characteristically the cell-wall material is very tough and extremely insoluble in a wide variety of solvents. It is made up to a large extent of sugar and amino acid components and presumably, therefore, falls into the class of 'mucoids' or 'mucosubstances' (Kent & Whitehouse, 1955). A similar conclusion was reached by Salton (1952, 1953) as a result of his findings in a smaller series of five Gram-positive and two Gram-negative species. One remarkable feature is the apparent simplicity of the amino acid patterns encountered, in contrast to the findings in other mucoids or in proteins.

Sugars and amino sugars were present in every preparation except those from *Staphylococcus aureus* and *S. albus*, in whose cell walls no hexoses or pentoses were detected, but these can be regarded as limiting cases. Glucosamine was invariably found, as was the unknown hexosamine-like substance already described in the section on methods. The exact nature of this substance is still obscure. Galactosamine, on the other hand, was present in only about one-third of the species examined. Among the sugars one or more of the three hexoses, glucose, galactose and mannose, seemed to be almost invariably present; arabinose and rhamnose occurred less frequently and had a more restricted range. Ribose has not been detected in cell-wall hydrolysates except in the case of some strains of corynebacteria. The amount was usually small and varied considerably in different preparations from the same strain, being entirely absent from some. Holdsworth (1952) did not detect ribose in the cell wall of *Corynebacterium diphtheriae*, and Salton (1953) did not find it in the wall of any of the seven species of bacteria he examined. It seems most likely, therefore, that the preparations in which ribose was present had not been adequately purified.

A very high proportion of the amino acid moiety of the cell-wall complex could in each case be accounted for in terms of three or four of the following amino acids: alanine, glutamic acid, lysine, α , ϵ -diaminopimelic acid, aspartic acid and glycine. Of these alanine and glutamic acid were invariably present as major components. The others were found to be characteristic major components of the cell walls of some organisms but not of others.

Apart from these major components, other amino acids were encountered in relatively smaller amounts in some of the preparations. We have never had any difficulty in deciding whether to call an amino acid a major or a minor component in any particular case, but the significance of the minor components is rather difficult to assess. They do not seem to be constant from one sample to another, and have generally been present only in traces except in the case of some preparations from corynebacteria examined early in the series, which showed moderately strong spots for lysine, serine, glycine, aspartic acid, valine and the leucines, as well as the characteristic major spots for alanine, glutamic acid and D.A.P. These corynebacterial preparations also contained a variable amount of ribose, and might legitimately be regarded as being contaminated by cytoplasmic remains. In other cases there were frequently no 'traces', for example, in hydrolysates from *Staphylococcus aureus* and *Lactobacillus plantarum*. It is not possible to decide at the moment whether these minor amino acid components are part of the cell-wall complex proper, or whether they

represent the remains of surface protein layers as exemplified by the M antigens of streptococci, or cytoplasmic remnants in slightly impure cell-wall suspensions.

The individual amino acids found are common ones, except for D.A.P., which has so far been described only in bacteria or their products (Work, 1951, 1955). The distribution of this substance among a variety of micro-organisms including bacteria, fungi, yeasts and protozoa was surveyed by Work & Dewey (1953), who examined hydrolysates of whole organisms. We have not noted any discrepancies between the distribution of D.A.P. as described by these workers in whole organisms of various species, and our own findings on separated cell walls and it seems possible that the bulk of the D.A.P. in Gram-positive bacteria is situated in the cell wall.

In the species we have examined, the cell walls contain either D.A.P. or lysine as a major component, but not both in similar quantities. This perhaps suggests that they have similar structural functions. Lysine can be formed from D.A.P. by the action of D.A.P. decarboxylase which is fairly widely distributed in bacteria (Dewey, 1954; Work, 1955), and it might have been expected that the decarboxylase would be found in those cases in which lysine and not D.A.P. was a major cell-wall component. There is, however, no such simple relationship, since some organisms which have lysine in their cell walls seem to be devoid of decarboxylase activity, while others which have D.A.P. apparently contain the enzyme (Work, 1955).

No account has been taken here of lipid components which may be present in the cell walls of any of the species examined. It would seem from the findings of Salton (1953) that this type of substance forms at most a relatively small part of the cell wall in Gram-positive bacteria. For example, he found 1.2 % total lipid in the cell walls of *Micrococcus lysodeikticus*, and 2.6 % in those of *Bacillus subtilis*. In both cases the lipid was firmly bound, and did not seem to be completely liberated until after several hours hydrolysis in 6 N-HCl at 100°.

Proteolytic enzymes are without effect on the cell-wall material as a whole, but may remove surface protein components such as the M antigens of streptococci (Salton, 1953). The removal of this material with trypsin made no difference to the appearance of the cell wall in electron micrographs, and did not seem to alter its physical properties, but Salton noted that the amino acid constitution was simpler after treatment with trypsin, and that in particular sulphur-containing and aromatic amino acids were no longer present. Cummins (1954) found that a superficial protein antigen in a strain of *Corynebacterium diphtheriae* appeared to be destroyed by pepsin, and it was this observation, together with Salton's findings in the case of the M antigen, that led us to adopt the routine use of trypsin followed by pepsin in the purification of the cell-wall material, in the hope of obtaining as simple a pattern of components as possible.

Cell-wall composition and bacterial taxonomy

The qualities necessary for a good taxonomic character have recently been discussed in some detail (Report of Discussion Meeting on the Principles of Microbial Classification, 1955), and the chemical composition of the bacterial

cell wall appears to fulfil in many respects the necessary requirements. The preparation of the purified cell-wall fractions, although somewhat laborious, is not technically difficult, and the components present can be accurately identified, if necessary, by comparison with synthetic standards. Cell-wall composition is also in our experience a stable character, unaffected by variations in culture media or conditions of growth, and this point is underlined by the close agreement between the present results and those of other workers. Our findings in the case of *Streptococcus faecalis* (NCTC 6782), for example, are virtually identical with those of Salton (1953) with the same strain, and there is the same degree of correspondence in the case of *Sarcina lutea*, although in this instance different strains were used.

In an attempt to test the stability of cell-wall composition under altered metabolic conditions, a strain of *Staphylococcus aureus* was 'trained' over a period of several months by growth in gradually increasing concentrations of penicillin in broth. The highly resistant strain so obtained would grow in the presence of 5000 units penicillin/ml., but its cell-wall composition was unaltered when compared with that of the original culture. It should be pointed out, however, that this resistant strain differed from those described by Gale & Rodwell (1948) and by Bellamy & Klimek (1948), in that it still retained the coccid form, and was still Gram-positive although somewhat irregularly so; it also grew both aerobically and anaerobically. The resistant strains described by the authors quoted appeared in stained films as pleomorphic Gram-negative bacilli, and would not grow anaerobically. They may therefore have undergone a more fundamental alteration than the strain described in the present paper.

Bacterial taxonomy is at present based on a number of different criteria, of which those of widest application are probably morphological appearances and staining properties, antigenic characteristics, and tests of biochemical activity which reflect various aspects of intermediary metabolism, mainly catabolic. Cell-wall composition can perhaps be regarded as an extension of morphology at the biochemical level; a sort of chemical anatomy. It is probably quite intimately connected with antigenic characteristics, since it is likely that important cell antigens are located in the cell wall, and indeed form a major part of it. It seems quite clear, for example, from the results of McCarty (1952*a, b*) and Salton (1953) that the cell walls of *Streptococcus pyogenes*, as prepared by mechanical disintegration, contain both the M protein and the C polysaccharide and since McCarty, and also Schmidt (1952), have shown that the latter is composed of rhamnose and hexosamine, it is not unlikely that it represents the polysaccharide moiety of the basic cell-wall substance in this species.

In the four main groups of Gram-positive bacteria so far examined (streptococci, corynebacteria, staphylococci and lactobacilli), the results of cell-wall analysis agree well with the genera already defined by the use of other taxonomic characters. The differences in composition seem clear cut and easily recognizable, and there would be no difficulty, for example, in distinguishing by this method a staphylococcus from a streptococcus, or a lactobacillus from

a corynebacterium. Figs. 1 and 2 show, in diagrammatic fashion, the pattern of constituents found in representative species of these four genera.

The general picture that emerges is one in which the amino acids present in the cell wall seem to be characteristic of the genus, and the sugars and amino sugars seem to characterize the species within the genus, with the important exception of arabinose and rhamnose, which appear to have special significance in *Corynebacterium* and *Streptococcus* respectively. In most cases only one strain of each species was examined, and the degree of species variation to be expected is not known. Where more than one strain has been analysed, the results have in some cases been virtually identical (e.g. *Corynebacterium diphtheriae*), while in others there have been definite qualitative differences in the sugar components present (e.g. *Lactobacillus plantarum*).

If it can be accepted that distinctive patterns of cell-wall constituents characterize different genera, then any organism which differs markedly in cell-wall composition from others in the genus to which it is normally assigned becomes of particular interest. In the present investigation three series of strains have given anomalous results of this sort, and they are considered below in turn.

In the case of *Corynebacterium pyogenes* and *C. haemolyticum* the results of cell-wall analysis are at variance with those obtained in other corynebacteria. The findings in the 6 strains of *C. pyogenes*, for example, would support the rejection of this species from the genus *Corynebacterium* and its inclusion instead in *Streptococcus*, since it contains rhamnose and not arabinose as a distinguishing cell-wall sugar, and has alanine, glutamic acid and lysine as major amino acid components, but lacks D.A.P. The same arguments apply also to the single strain of *C. haemolyticum* examined. There is no doubt that these organisms would be included with the corynebacteria, if morphology alone were considered, although in some conditions they can be almost coccal. On the other hand, McLean *et al.* (1946), in their original description of *C. haemolyticum*, commented on the close resemblance of this species to *Streptococcus pyogenes*, and the growth of *Corynebacterium pyogenes* on blood agar and in broth resembles very closely that of a β -haemolytic streptococcus. In addition, both species resemble the streptococci in being catalase-negative, while other corynebacteria are catalase-positive. It seems to us, therefore, that there are good grounds for reconsidering the taxonomic position of *C. pyogenes* and *C. haemolyticum*, with a view to their inclusion in the genus *Streptococcus*.

The results of cell-wall analysis in the case of the four strains of *Lactobacillus plantarum* present a more difficult problem. The amino acid pattern in these strains is quite different from that of the other lactobacilli examined, and is in fact identical with the pattern found in *Corynebacterium*, and with that which a few preliminary observations suggest may also be found in the genus *Bacillus* (Salton, 1953; Cummins & Harris, unpublished observations). Quite apart from any morphological and physiological differences, however, these four strains of *Lactobacillus plantarum* were distinguished from the corynebacteria by the fact that none of them contained arabinose as a major cell-wall component, nor is there any evidence to suggest any close relationship to the

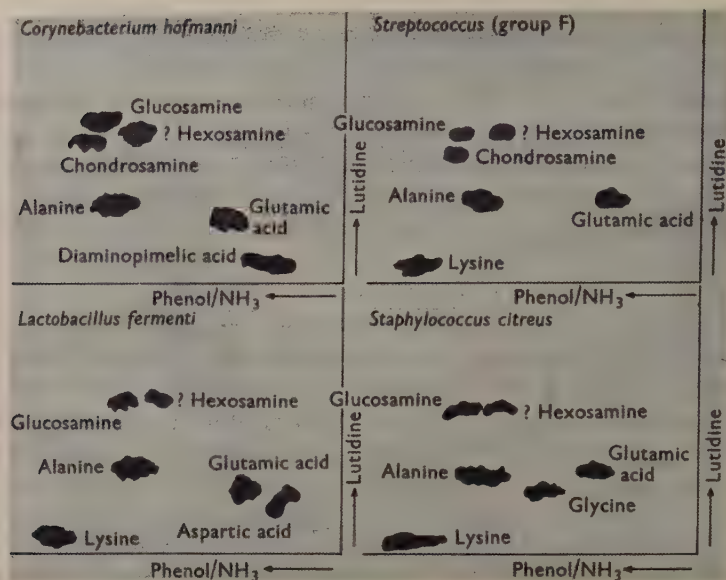


Fig. 1. Diagrams of typical chromatograms showing the amino acid and amino sugar patterns in representative species of four different genera.

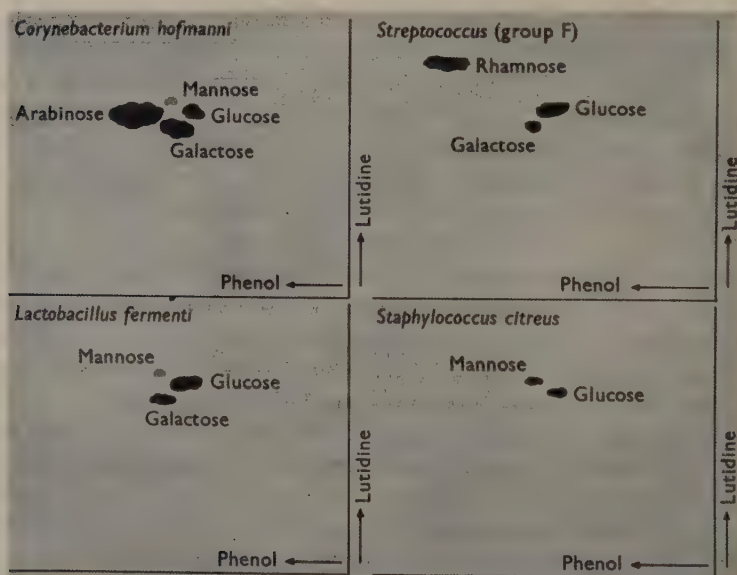


Fig. 2. Diagrams of typical chromatograms showing the sugar patterns in representative species of four different genera.

aerobic sporing bacilli. More than eighty strains of *Lactobacillus plantarum*, together with other species of lactobacilli, have recently been investigated by physiological and serological methods (Wheater 1955*a, b*; Briggs 1953; Sharpe, 1955), and the results obtained did not suggest that the position of *L. plantarum* in this genus was anomalous (Wheater, personal communication). It is interesting, however, to note that Camien (1952) found D-aspartic acid to be an essential metabolite for *L. brevis* and *L. lycopersici*, while it was not required by a strain of *L. plantarum*. Furthermore, D-aspartic acid could not be detected in hydrolysates of the latter species, although significant amounts of it were found in the other lactobacilli. The significance of the unusual cell-wall composition of *L. plantarum* may become apparent when a wider survey of other Gram-positive bacilli has been undertaken.

With regard to the two strains labelled *Micrococcus rhodochrous* and *M. cinnabareus* (Table 3), reasons have already been given for thinking that these organisms belong to the genus *Corynebacterium*. It seems possible that the same consideration might apply to various other strains at present classified as *Rhodococcus*.

We are indebted to Professor C. F. Barwell, Professor F. L. Warren and Dr S. T. Cowan for reading the manuscript, and to Dr Cowan also for much helpful advice on points of bacterial taxonomy; to Dr Dorothy Wheeler for information about physiological tests in the classification of lactobacilli; to Dr D. Payne, Dr W. H. H. Jebb Dr F. C. O. Valentine, Dr Lane Barksdale and Mr J. D. Paterson for providing cultures for examination; to Mr R. C. Valentine, National Institute for Medical Research, Mill Hill, for electron micrographs; and finally to Mr B. Cohen for his excellent and painstaking technical assistance.

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The Continuous Culture of Bacteria; a Theoretical and Experimental Study

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SUMMARY: A theoretical treatment of continuous culture is given, which allows quantitative prediction of the steady-state concentrations of bacteria and substrate in the culture, and how these may be expected to vary with change of medium, concentration and flow-rate. The layout and operation of a small pilot plant for the continuous culture of bacteria are described. This plant has been operated continuously for periods of up to 4 months without breakdown or contamination of the culture. No alterations in the properties of the organisms studied have occurred during such periods of continuous culture. Results are given of a series of experiments on the continuous culture of *Aerobacter cloacae* in a chemically defined medium, designed to allow quantitative comparison with the results predicted by the theory. The relative advantages of batch and continuous culture as production processes are discussed, and it is concluded that continuous culture may usually be expected to show a five to tenfold increase in output as compared with a batch process.

The continuous culture of micro-organisms is a technique of increasing importance in microbiology. The essential feature of this technique is that microbial growth in a continuous culture takes place under steady-state conditions; that is, growth occurs at a constant rate and in a constant environment. Such factors as pH value, concentrations of nutrients, metabolic products and oxygen, which inevitably change during the 'growth cycle' of a batch culture, are all maintained constant in a continuous culture; moreover, they may be independently controlled by the experimenter. These features of the continuous culture technique make it a valuable research tool, while it offers many advantages, in the form of more economical production techniques, to the industrial microbiologist. Nevertheless, the technique has so far been comparatively little used. (The review of Novick (1955) lists nearly all the work on the subject that has yet appeared.) The reasons for this relative neglect are, we believe, twofold.

The first reason is the lack of a generally accepted theoretical background. Continuous culture presents theoretical problems of an essentially kinetic nature which must be solved before the technique can be intelligently applied. The basis of a correct theoretical treatment has, in fact, already been laid in important papers by Monod (1950) and Novick & Szilard (1950), but later writers on the subject (Golle, 1953; Finn & Wilson, 1954; Northrop, 1954) have either disagreed with, or failed to understand, this work; at least their theoretical treatments are quite different (and in our view erroneous). This may have been because the earlier writers presented little experimental data in support of their theoretical conclusions. One object of the present work was to obtain quantitative data to test the general validity of Monod's theory.

A second reason for the neglect of continuous culture techniques, in particular by industrial microbiologists, is the apparently widespread belief that they are so difficult as to be impracticable. Difficulty in maintaining sterility during long runs and the probability of mutations are two objections which have frequently been raised (e.g. Warner, Cook & Train, 1954*a, b*). These objections, which have been answered by Dawson & Pirt (1954), are based solely on conjecture since no serious attempts have hitherto been made (in Great Britain at least) to apply continuous techniques in industrial microbiology. Our own experience shows that these purely practical difficulties have been greatly exaggerated.

The present paper attempts to deal with both of the aspects of continuous culture mentioned above. In the first part a mathematical theory of continuous culture is presented; the second part describes the operation of a small pilot-scale continuous culture apparatus and gives data obtained during continuous growth of *Aerobacter cloacae*, for comparison with results predicted by theory.

THEORY

General principles of continuous flow systems

All continuous flow systems consist essentially of some form of reactor into which reactants flow at a steady rate and from which products emerge. The factors governing their operation are: (i) the way in which material passes through the reactor (which depends upon its design); (ii) the kinetics of the reaction taking place. As Danckwerts (1954) stated, (i) may be characterized by the *distribution of residence-times* of molecules or minute particles passing through the system. Most reactors lie between the extremes of the completely-mixed tank and the ideal tubular type with 'piston flow' and no mixing. In ideal piston flow all particles have the same residence-time, equal to the *mean residence-time*, \bar{t} , while complete mixing produces a wide spread of residence-times about the mean.

In a piston-flow reactor the extent of the reaction (whatever its kinetics) will be the same as for a batch reactor operated for a period equal to \bar{t} , while in a completely mixed reactor some of the material will have reacted for a considerably longer and some for a considerably shorter period than \bar{t} . Danckwerts (1954) pointed out that the piston flow reactor will be the more efficient for chemical reactions whose rates fall off as the reaction proceeds, but the completely-mixed reactor will be more efficient for reactions of the 'autocatalytic' type whose rate increases with time. Since bacterial growth is an autocatalytic process, the completely-mixed reactor will therefore be the most efficient type for continuous bacterial culture (a fact which has not always been appreciated), and only this type will be considered here.

Completely-mixed continuous culture apparatus. In the type of apparatus to be considered the reactor consists of some form of culture vessel in which the organism can be grown under suitable conditions. Sterile growth medium is fed into the culture vessel at a steady flow-rate f and culture emerges from it at the same rate, a constant-level or similar device keeping the volume of culture in

the vessel (v) constant. The contents of the vessel are sufficiently well stirred to approximate to the ideal of complete mixing, so that the entering growth medium is instantaneously and uniformly dispersed throughout the vessel.

Residence-times in such a culture vessel will be determined not by the absolute values of the flow-rate and culture volume but by their ratio which we call the *dilution rate*, D , defined as $D=f/v$, i.e. the number of complete volume-changes/hr. The mean residence-time of a particle in the culture vessel is evidently equal to $1/D$.

Assume for the moment that the bacteria in the culture vessel are not growing or dividing. With complete mixing, every organism in the vessel has an equal probability of leaving it within a given time. It can easily be shown to follow that the fraction of the total organisms in the vessel having a residence-time $\geq t$ is e^{-Dt} . The *wash-out rate*, i.e. the rate at which organisms initially present in the vessel would be washed out if growth ceased but flow continued is therefore:

$$-\frac{dx}{dt} = Dx, \quad (1)$$

where x is the concentration of organisms in the vessel. The distribution of residence-times and the wash-out rate in a completely-mixed continuous culture vessel of this sort can thus be adequately described in fairly simple terms. Their application to continuous culture requires, in addition, a knowledge of the basic kinetics of the growth process.

Kinetics of bacterial growth

Theoretical discussions of bacterial growth usually start from the familiar 'exponential growth' equation

$$\frac{1}{x} \frac{dx}{dt} = \frac{d(\log_e x)}{dt} = \mu = \frac{\log_e 2}{t_d}, \quad (2)$$

where x is the concentration of organisms (dry weight of organisms/unit volume) at time t , μ is the *specific growth rate** and t_d is the *doubling time*,† i.e. the time required for the concentration of organisms to double. In this equation, μ and t_d are usually assumed to be constants; it is insufficiently appreciated, however, that this assumption is correct only when all substrates necessary for growth are present in excess.

Monod (1942) first showed that there is a simple relationship between the specific growth rate and the concentration of an essential growth substrate, μ being proportional to the substrate concentration when this is low but reaching a limiting saturation value at high substrate concentrations according to the equation

$$\mu = \mu_m \left(\frac{s}{K_s + s} \right), \quad (3)$$

* The actual rate of increase of concentration of organisms (dx/dt) is called the *growth rate*; the rate of increase/unit of organism concentration ($\frac{1}{x} \frac{dx}{dt}$) is called the *specific growth rate*.

† The doubling time is sometimes confused with the *mean generation time*, but strictly the two could only be identical if all organisms had identical individual generation times.

where s is the substrate concentration, μ_m is the *growth rate constant* (i.e. the maximum value of μ at saturation levels of substrate) and K_s is a *saturation constant* numerically equal to the substrate concentration at which $\mu = \frac{1}{2} \mu_m$. It follows from equation (3) that exponential growth can occur at specific growth rates having any value between zero and μ_m , provided the substrate concentration can be held constant at the appropriate value—a fact of major importance in continuous culture (Monod, 1950; Novick & Szilard, 1950).

Monod (1942) also showed that there is a simple relationship between growth and utilization of substrate. This is shown in its simplest form in growth media containing a single organic substrate (e.g. glucose, ammonia and salts); under these conditions the growth rate is a constant fraction, Y , of the substrate utilization rate:

$$\frac{dx}{dt} = -Y \frac{ds}{dt}, \quad (4)$$

where Y is known as the *yield constant*. Thus over any finite period of growth

$$\frac{\text{weight of bacteria formed}}{\text{weight of substrate used}} = Y.$$

If the values of the three growth constants μ_m , K_s and Y are known, equations (2) to (4) provide a complete quantitative description of the 'growth cycle' of a batch culture (Monod, 1942). The same equations and constants are equally applicable to the theoretical treatment of continuous culture.

Growth in continuous culture

Experimental arrangement. Consider bacteria growing in a completely-mixed type of continuous culture vessel as described above, the inflowing medium containing a single organic substrate (e.g. glucose) at a concentration s_R . It is assumed that all other substrates are present in excess, and the culture vessel is so efficiently aerated that the oxygen supply is always adequate; the supply of organic substrate is therefore the sole growth-limiting factor. (Alternatively, conditions may be assumed to be completely anaerobic, when the same theoretical treatment will apply, though the actual values of μ_m and Y will be different.) The variables within the control of the experimenter are the substrate concentration and flow rate of the incoming culture medium and a complete theory must describe how variation of these affects the growth rate and the concentrations of organisms and of substrate in the growth vessel.

Changes in concentration of organisms. In the culture vessel the organisms are growing at a rate described by equation (2) and simultaneously being washed away at a rate determined by equation (1). The net rate of increase of concentration of organisms is given by the simple balance equation (individual terms referring to *rates* in each case):

$$\text{increase} = \text{growth} - \text{output},$$

or

$$\frac{dx}{dt} = \mu x - Dx. \quad (5)$$

Hence if $\mu > D$, dx/dt is positive and the concentration of organisms will increase, while if $D > \mu$, dx/dt is negative and the concentration of organisms will decrease, eventually to zero; i.e. the culture will be 'washed out' of the culture vessel. When $\mu = D$, $dx/dt = 0$ and x is constant; i.e. we have a steady state in which the concentration of organisms does not change with time. Under such steady-state conditions, the specific growth rate, μ , of the organisms in the culture vessel is exactly equal to the dilution rate D . This equation has been derived by several workers, but it has not always been realized that by itself the equation gives no information on what dilution rates make a steady state possible. To know this, we must also know how dilution rate affects the concentration of substrate in the culture vessel, since as already mentioned (equation 3) the value of μ depends on s .

Changes in substrate concentration. In the culture vessel, substrate is entering at a concentration s_R , being consumed by the organisms and flowing out at a concentration s . The net rate of change of substrate concentration is obtained by another balance equation (individual terms again referring to rates):

increase = input - output - consumption

$$= \text{input} - \text{output} - \frac{\text{growth}}{\text{yield constant}} \quad (\text{from eqn. 4}),$$

$$\frac{ds}{dt} = Ds_R - Ds - \frac{\mu x}{Y}. \quad (6)$$

Fundamental equations of continuous culture. Equations (5) and (6) both contain μ , which is itself a function of s (equation 3). Substituting (3) in these equations we have:

$$\text{from (5)} \quad \frac{dx}{dt} = x \left\{ \mu_m \left(\frac{s}{K_s + s} \right) - D \right\}, \quad (7)$$

$$\text{and from (6)} \quad \frac{ds}{dt} = D(s_R - s) - \frac{\mu_m x}{Y} \left(\frac{s}{K_s + s} \right). \quad (8)$$

These are virtually identical with equations given by Monod (1950), though the above derivation is different from (and, we think, simpler than) Monod's. They define completely the behaviour of a continuous culture in which the fundamental growth relations are given by equations (2) to (4).

The steady state. It is apparent from consideration of equations (7) and (8) that if s_R and D are held constant and D does not exceed a certain critical value (see equation 12), then unique values of x and s exist for which both dx/dt and ds/dt are zero; i.e. the system is in a steady state. Solving (7) and (8) for $dx/dt = ds/dt = 0$, these steady-state values of x and s , which will be designated \tilde{x} and \tilde{s} are given as

$$\tilde{s} = K_s \left(\frac{D}{\mu_m - D} \right), \quad (9)$$

$$\tilde{x} = Y(s_R - \tilde{s}) = Y \left\{ s_R - K_s \left(\frac{D}{\mu_m - D} \right) \right\}. \quad (10)$$

From these equations the steady-state concentrations of bacteria and substrate in the culture vessel can be predicted for any value of the dilution rate and concentration of inflowing substrate, provided the values of the growth constants μ_m , K_s and Y are known. They may be said to describe completely the behaviour of a continuous culture running under steady-state conditions. These equations also were first derived by Monod (1950).

While these equations describe accurately the situation existing once a steady state has become established, no proof was given by Monod that, starting from non-steady state conditions (such as exist at inoculation for example), a steady state must inevitably be reached. Rigorous proof of this has lately been provided by our colleague E. O. Powell who has shown (unpublished work) that, starting from any initial values of x and s , the system inevitably adjusts itself to the steady state defined by equations (9) and (10), and that this is the only stable state of the system. For example, consider a system which has just been inoculated, when x is very small, s is nearly equal to s_R and $\mu > D$. The concentration of organisms will increase, but owing to the resulting fall in substrate concentration the specific growth rate will decrease, until eventually μ becomes equal to D . At this point the combined rates of substrate consumption and loss just balance the rate of substrate addition and the system shows no further tendency to change. The system is stable in the sense that small accidental fluctuations from the steady-state values will set up opposing reactions which will restore the *status quo*. It is this automatic self-adjusting property of the system that makes continuous culture a readily feasible possibility.

As previously mentioned, in the steady-state the specific growth rate is equal to the dilution rate

$$\mu = \frac{\log_e 2}{t_d} = \mu_m \left(\frac{\tilde{s}}{K_s + \tilde{s}} \right) = D. \quad (11)$$

The doubling time t_d is therefore equal to $0.693/D$; e.g. if one volume per hour is flowing through the culture vessel, the mass of organisms will be doubling every 42 min.

As is evident from equations (9) and (10), the steady-state values of the concentrations of organisms and substrate depend solely on the values of s_R and D (since μ_m , K_s and Y are constant for a given organism and growth medium). By varying s_R and D an infinite number of steady states can be obtained.

Effect of varying dilution rate. Fig. 1 shows how the mean generation time and the steady-state concentrations of bacteria and substrate in a continuous culture may be expected to vary when the dilution rate is varied, the inflowing substrate concentration being held constant; the curves are plotted from equations (9) and (10). The concentration of organisms has a maximum value when the dilution rate is zero, the substrate concentration then also being zero; i.e. the situation corresponds to the final stage of a batch culture. As the dilution rate increases the substrate concentration increases and the concentration of organisms falls, until at a critical value of D the concentration of organisms becomes zero and the substrate concentration becomes equal to s_R ;

if plotted on appropriate scales the curves for bacterial and substrate concentrations are mirror-images.

The critical value of the dilution rate, above which complete 'wash-out' occurs, is obviously of great practical importance. This critical value, which is designated D_c , will be seen from equation (5) to be equal to the highest possible value of μ , which is the value attained when \tilde{s} has its highest possible value s_R , and is given by

$$D_c = \mu_m \left(\frac{s_R}{K_s + s_R} \right). \quad (12)$$

When $s_R \gg K_s$, which is usually the case, then $D_c \sim \mu_m$.

At all dilution rates greater than D_c , dx/dt will be negative (from equation 5), bacteria will be washed out of the culture vessel faster than they can grow, and no steady state with $\tilde{x} > 0$ is possible.

Effect of inflowing substrate concentration. In Fig. 2 the variation with the dilution rate of the steady-state concentrations of organisms and substrate is shown for a number of different values of the inflowing substrate concentration s_R ; the curves are plotted from equation (9). It will be seen that at a given dilution rate below the critical the concentration of organisms is nearly proportional to s_R (see equation 9), but the concentration of substrate is independent of s_R , i.e. the curve relating dilution rate to the concentration of substrate in the culture vessel is the same whatever the concentration of substrate in the inflowing culture medium (cf. Novick & Szilard, 1950). In other words, when the dilution rate is fixed, the substrate concentration must come to a level (determined by equation 9) that makes μ equal to D , and this level is independent of s_R .

The curve relating concentration of organisms to dilution rate is seen (Fig. 2) to be displaced vertically as s_R increases, the drop in \tilde{x} at high dilution rates being steeper for higher values of s_R . Consideration of equation (3) shows that the important factor here is not the absolute value of s_R but the value of the ratio s_R/K_s . The higher this ratio, the greater the fraction of total substrate that can be consumed without an appreciable decrease in the specific growth rate. Hence as s_R/K_s is increased the concentration of organisms is maintained at nearly the maximum value up to higher values of D and the critical dilution rate D_c approaches more closely to μ_m (cf. equation 12). The value of K_s is very low (10^{-4} M or less) for most substrates, so that s_R/K_s ratios will usually be high in practice.

Performance criteria: output and yield. When a continuous culture system is viewed as a production process, its performance may be judged by two criteria: (i) the quantity of bacteria produced in unit time, which will be called the *output rate*; (ii) the quantity of bacteria produced from unit weight of substrate, which will be called the *effective yield*.

The total output from a continuous culture unit in the steady state is obviously equal to the product of flow-rate and concentration of organisms, or $f\tilde{x}$; the output/unit volume of culture is therefore $D\tilde{x}$, and from equation (10):

$$\text{Output} = D\tilde{x} = DY \left\{ s_R - K_s \left(\frac{D}{\mu_m - D} \right) \right\}. \quad (13)$$

Now as D is increased from 0 to D_c , \tilde{x} decreases from Ys_R to 0, and it can be shown that there is a value of D for which the product $D\tilde{x}$ is a maximum; in other words, for any system there is a particular dilution rate, D_M , which gives the maximum output of organisms in unit time. This maximum output value of D is obtained by differentiating equation (13) with respect to D and equating to zero, and is

$$D_M = \mu_m \left\{ 1 - \sqrt{\frac{K_s}{K_s + s_R}} \right\}. \quad (14)$$

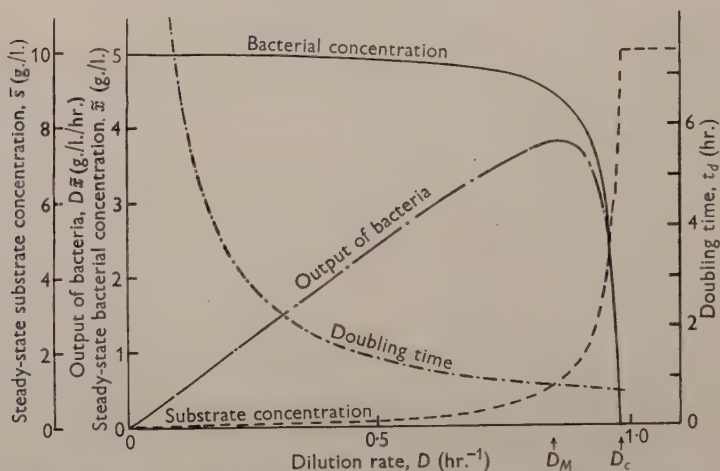


Fig. 1. Steady-state relationships in a continuous culture (theoretical). The steady-state values of substrate concentration, bacterial concentration and output at different dilution rates are calculated from equations (9) and (10), for an organism with the following growth constants: $\mu_m = 1.0 \text{ hr.}^{-1}$, $Y = 0.5$ and $K_s = 0.2 \text{ g./l.}$; and a substrate concentration in the inflowing medium of $s_R = 10 \text{ g./l.}$

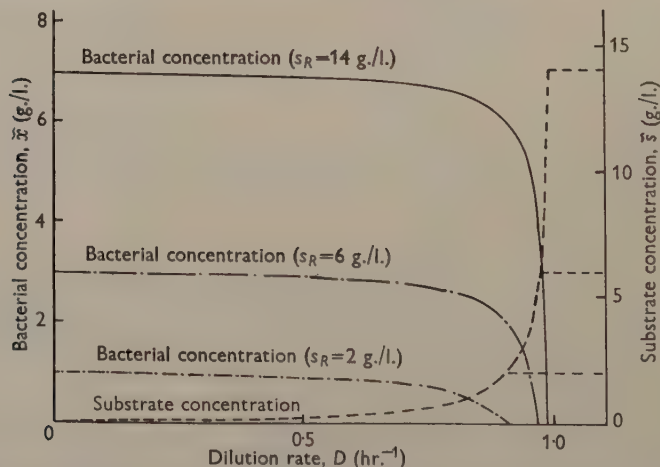


Fig. 2. Effect of varying the concentration of substrate in the inflowing medium (s_R) on the steady-state relationships in a continuous culture (theoretical). The curves are calculated from equations (9) and (10) for an organism with $\mu_m = 1.0 \text{ hr.}^{-1}$, $Y = 0.5$, and $K_s = 0.1 \text{ g./l.}$, for media of three different substrate concentrations.

The steady-state concentration of organisms at this dilution rate is obtained by substituting the above value of D_M in equation (10) and is

$$\tilde{x}_M = Y \{ (s_R + K_s) - \sqrt{(K_s(s_R + K_s))} \}. \quad (15)$$

The *maximum output rate*, $D_M \tilde{x}_M$, is the product of equations (14) and (15) and is given below (equation 23).

Output of organisms is plotted against dilution rate in Fig. 1. The output is nearly proportional to D at low D values (up to about $0.5 D_c$); as D increases the output curve flattens, reaches a maximum and falls rapidly to zero at the critical dilution rate.

The yield constant Y , defined as in equation (4), is related to the steady-state concentrations of organisms and substrate in a continuous culture by the expression

$$Y = \frac{\text{output of bacteria}}{\text{substrate utilized}} = \frac{\tilde{x}}{s_R - \tilde{s}}. \quad (16)$$

The *effective yield*, Y_E , is defined as the ratio of bacteria formed to substrate supplied in the inflowing culture medium and is given by

$$Y_E = \frac{\text{output of bacteria}}{\text{input of substrate}} = \frac{\tilde{x}}{s_R} = \frac{Y(s_R - \tilde{s})}{s_R}. \quad (17)$$

At all flow-rates > 0 , the effective yield is less than the yield constant owing to the substrate wasted in the outflow, \tilde{s} , which increases with the dilution rate (Fig. 1).

The efficiency of utilization of the substrate supplied in the inflowing growth medium is given by equations (9) and (17) as

$$\frac{Y_E}{Y} = \frac{s_R - \tilde{s}}{s_R} = \frac{s_R - K_s \left(\frac{D}{\mu_m - D} \right)}{s_R}. \quad (18)$$

It follows that for maximum utilization of substrate the dilution rate should be as low as possible; this is, strictly speaking, incompatible with a maximum output of bacteria, which requires the dilution rate to be high (D_M being close to D_c). However, the shape of the curve relating \tilde{s} to D (Fig. 1) is such that loss of substrate in the outflow is in practice negligible up to quite high dilution rates; in the example plotted utilization of substrate is $> 95\%$ complete at all dilution rates up to $0.7 D_c$, and is still 90% complete at the maximum output rate D_M .

The efficiency of utilization of substrate at the maximum output rate D_M is given by equations (14) and (18) as

$$\frac{s_R - \tilde{s}_M}{s_R} = \frac{(s_R/K_s + 1) - \sqrt{(s_R/K_s - 1)}}{s_R/K_s}. \quad (19)$$

The efficiency of utilization will be seen to depend solely on the ratio s_R/K_s and approaches 100% if this ratio is made sufficiently high; in other words, high substrate concentrations are advantageous for efficient utilization of substrate.

To summarize, the conditions for maximum production efficiency, combining a high output with efficient utilization of substrate, will be obtained

with a flow-rate at or a little below the maximum output rate D_M and the highest practicable substrate concentration. It must be emphasized, however, that these are the optimum conditions when the object is to produce micro-organisms. When the desired product is a fermentation product whose formation is proportional to the amount of substrate breakdown (e.g. ethanol, lactic acid), optimum conditions should be much the same, but they may be widely different for the production of complex metabolic products such as antibiotics or exotoxins.

Comparison of continuous and batch culture. The relative outputs of continuous and batch cultures are of interest from the production standpoint. The output of a batch culture of course varies throughout the growth cycle, but a mean output can be calculated as follows.

Consider a batch of medium of initial substrate concentration s_0 , inoculated initially with organisms to a concentration x_0 ; the maximum growth attained when all substrate has been utilized is x_m . Then the total time of one production cycle is

$$t = \frac{1}{\mu_m} \log_e \frac{x_m}{x_0} + t_L, \quad (20)$$

where the first term is the time which would be occupied if the organisms grew exponentially at maximum rate from start to finish, and the second term t_L is an overall 'delay time' which includes the initial lag and final retardation phases of growth and the 'turnaround time' necessary to take down, sterilize and re-assemble the plant preparatory to a second cycle.

The total amount of organisms produced (from equation 3) is

$$x_m - x_0 = Y s_0 \left(\frac{x_m - x_0}{x_m} \right). \quad (21)$$

The mean output is therefore

$$\frac{\text{total organisms produced}}{\text{total cycle time}} = \frac{\mu_m Y s_0 \left(\frac{x_m - x_0}{x_m} \right)}{\log_e \frac{x_m}{x_0} + \mu_m t_L} \quad (22)$$

The maximum output of a continuous culture is from equations (14) and (15):

$$D_M \tilde{x}_M = \mu_m Y s_R \left\{ \sqrt{\frac{K_s + s_R}{s_R}} - \sqrt{\frac{K_s}{s_R}} \right\}^2, \quad (23)$$

where the term in brackets has a value close to 1.0 for high s_R/K_s ratios such as obtain in practice and may therefore be neglected. Hence, when the same growth medium is used in both cases, so that $s_0 = s_R$, we have from equations (22) and (23)

$$\frac{\text{continuous output}}{\text{batch output}} = \frac{\log_e \frac{x_m}{x_0} + \mu_m t_L}{(x_m - x_0)/x_m} \quad (24)$$

The value of this ratio is affected mainly by the growth rate of the organism and the total delay time, inoculum size having a lesser effect. Assuming

a '5 % inoculum' ($x_0/x_m = 0.05$), and a delay time t_L of 6 hr., calculations from (24) give the following results:

Doubling time of organism hr.	Ratio: continuous output batch output
0.5	11.9
1.0	7.6
2.0	5.3
4.0	4.3

The delay time of 6 hr. assumed in these calculations is fairly optimistic; both plant turn-around time and growth lag would exceed this in many instances, which would further increase the above ratios. In the majority of cases, therefore, a continuous process would be expected to show at least a five- to tenfold advantage over the corresponding batch process.

APPARATUS AND METHODS

Continuous culture apparatus. The apparatus used is of conventional small pilot plant type and is fabricated wholly in stainless steel; the layout is shown in Fig. 3. It consists of two sterilizing tanks S_1 , S_2 (each of 300 l. working volume) connected in parallel and used alternately to feed growth medium into the culture vessel, CV . This has a working capacity of 20 l. and is of conventional design; mixing and aeration are effected by a vane-disk impeller mounted on a central shaft passing through a stuffing-box in the lid, with wall baffles and injection of sterile air immediately under the impeller, similar to the arrangement of Chain *et al.* (1954). The oxygen transfer rate, measured by the method of Cooper, Fernstrom & Miller (1944), is 135 mmole O_2 /l./hr. with an air flow of 1.0 vol./vol. culture/min. The level of culture in the vessel is maintained constant by a side overflow tube through which the culture flows by gravity into two calibrated collecting tanks M_1 , M_2 , which are connected in parallel, and thence to a large holding tank H . Effluent air leaves the culture vessel by a separate outlet; both entering and exit air is sterilized by heat. Air and medium flow-rates are indicated by Rotameters R_1 , R_2 , and are controlled by manual adjustment of cocks; a second check on medium flow-rate is provided by the calibrated tanks M_1 , M_2 . Sampling points SP_{1-2} , SP_3 , are provided on the sterilizers and culture vessel respectively. On the culture vessel there is a point AP_1 for the addition of inoculum and anti-foam ('Anti-foam A', Midland Silicones Ltd.). Temperature is controlled by circulating thermostatically controlled water through an internal coil (not shown in diagram) in the culture vessel and was 37° for all experiments described in this paper.

Operation. The whole equipment is sterilized empty with internal steam at 20 lb./sq.in., followed by filling and sterilization of the medium reservoirs. The culture vessel is then charged with 20 l. of sterile medium and inoculated with 1 l. of a shake-flask culture of the selected organism grown on the same medium. Growth is allowed to proceed batchwise until the concentration of organisms

reaches 90 % of the expected peak value; medium flow through the culture vessel is then started at a selected value and the culture from then on run continuously. Samples of sterile medium are taken from each sterilizing tank for analysis and samples of the culture are usually taken hourly; samples of the exit air are also taken for gas analysis.

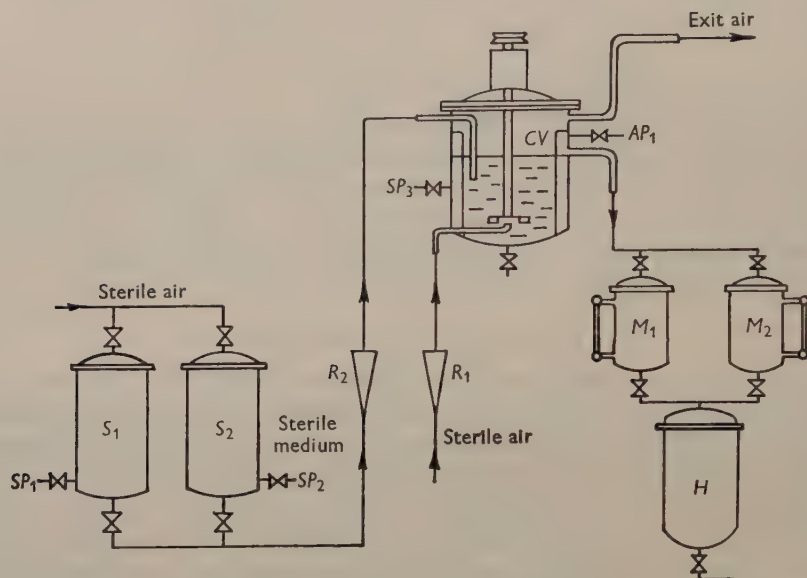


Fig. 3. Pilot plant for continuous culture of bacteria (schematic). S_1 , S_2 , sterilizing tanks (300 l. working volume); CV culture vessel (20 l. working volume); M_1 , M_2 , measuring tanks (30 l.); H , holding tank; R_1 , R_2 , rotameters; SP_1 , SP_2 , SP_3 , sampling points; AP_1 , anti-foam addition point.

Observations and measurements. Each batch of medium was examined or analysed for sterility, pH value, ammonia-N content and concentration of organic substrate, which in the present instance was glycerol, determined according to Neish (1952). Samples from the culture vessel were tested for pH value, concentration of glycerol, total count (by Helber counting chamber), viable count (by the Miles & Misra (1938) technique on plates of Hartley's digest agar) and purity of the microbial species being grown.

Organisms and culture medium. *Aerobacter cloacae* (*Cloaca cloacae* strain NCTC 8197) was used throughout and grown in medium of the following composition: 0.09 M- $(\text{NH}_4)_2\text{HPO}_4$, 0.01 M- NaH_2PO_4 , 0.01 M- K_2SO_4 , 0.001 M- MgSO_4 , 0.0001 M- CaCl_2 , 0.00002 M- FeSO_4 , and 0.0272 M (0.25 %, w/v) glycerol; the pH value after sterilizing was 7.3–7.4. In this medium glycerol (selected in preference to glucose for its stability on sterilization) was the sole carbon source and was also the growth-limiting component, all other components being present in excess. A rather low concentration was used to ensure fairly low bacterial concentrations so that aeration was always adequate.

RESULTS

Growth in batch culture

As a preliminary to continuous culture studies, data were obtained on the growth of the organism in batch culture, using the same apparatus and growth medium. Typical experiments are shown in Fig. 4. After a variable lag period, which depends on the age and size of the inoculum, growth proceeds exponentially until almost all the substrate has been exhausted (a sign that aeration is not rate-limiting). Under these conditions the organisms are growing for most of the time in substrate concentrations which are high compared with K_s , and equation (3) becomes

$$\frac{1}{x} \frac{dx}{dt} = \frac{d(\log_e x)}{dt} \sim \mu_m. \quad (25)$$

The value of μ_m is given by the slope of the straight line obtained by plotting $\log_e x$ against t , the best straight line being fitted by the 'method of least squares'. The mean value obtained from a number of experiments was $\mu_m = 0.85 \text{ hr.}^{-1}$ (doubling time $t_d = 49 \text{ min.}$).

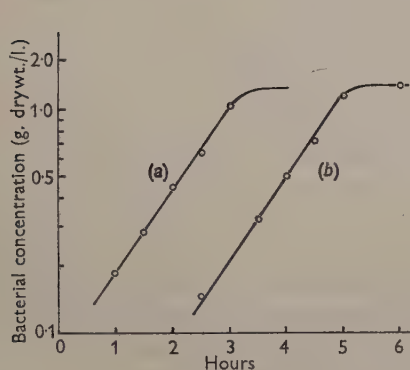


Fig. 4

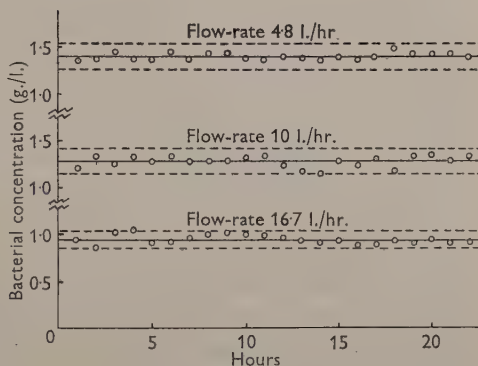


Fig. 5

Fig. 4. Growth curves of *Aerobacter cloacae* in batch culture. Details of growth conditions and culture medium are given in the text. Curve (a) was obtained from a seed culture at the start of a continuous culture run; curve (b) was obtained after the culture had been operated continuously for 6 weeks (the points on curve (b) have been displaced 2 hr. to the right to avoid overlapping with curve (a)).

Fig. 5. Steady states in continuous culture. *Aerobacter cloacae* was grown in continuous culture as described in the text; the data plotted are hourly measurements of bacterial concentration at three different flow-rates. The continuous lines are the lines of best fit, calculated statistically; the dotted lines are the 95 % fiducial limits.

In the medium used, the total growth ($x_m - x_0$) obtained in a batch culture was strictly proportional to the initial concentration of glycerol in the medium (cf. Monod, 1942). The mean of several determinations of the yield constant was $Y = 0.53 \text{ g. dry weight of organism/g. glycerol used}$; this corresponds to a 61 % conversion of the glycerol carbon to bacterial carbon. (The carbon content of the bacteria was found to be 45 % of the dry weight.)

Growth in continuous culture

Steady-state operation. In accordance with theory, steady-state operation was found to be possible over a wide range of flow-rates, the range actually tested being from 4 to 22 l./hr., corresponding to dilution rates of 0.2–1.1 hr.⁻¹. The critical flow-rate for complete wash-out (obtained by extrapolation of the curves of Fig. 6) was about 24 l./hr., corresponding to a critical dilution rate of about 1.2 hr.⁻¹.

Over the stable range of flow-rates the culture was self-adjusting; i.e. on setting the flow-rate to a given value, the concentrations of organisms and substrate would move towards and settle down at steady levels which were maintained indefinitely so long as the flow-rate remained unaltered; on changing the flow-rate, new steady-state levels were automatically attained. After a change of flow-rate, some hours might elapse before the culture had stabilized at the new steady state, particularly when the change in D was large. The culture was therefore always run for at least 24 hr. after a flow-rate change, before measurements at the new steady state were begun.

Examples of steady states at three different flow-rates are given in Fig. 5; measurements of bacterial concentration were taken hourly for 24 hr., after a preliminary 24 hr. stabilization period. Statistical analysis of the results showed that the plots of bacterial concentration against time did not differ significantly from straight lines of zero slope; the apparent small fluctuations in bacterial concentration were purely random and within the errors of measurement (95 % fiducial limits for each curve are shown by the dotted lines).

Maintenance of purity of culture. Under this heading we include both contamination of the culture with foreign micro-organisms and mutation of the parent organism to an extent sufficient to alter the characteristics of the culture. To detect such occurrences, samples taken directly from the culture vessel were repeatedly examined (a) microscopically, (b) by plating out and examination of colonies, (c) by regular subculturing and examination by biochemical tests of typical colonies (and any atypical ones, when observed).

Such tests readily detect gross contamination, but statistical considerations show that they are not very efficient in revealing small degrees of contamination. Suppose that in the whole culture there is an average of z contaminants to every n total organisms. If z is small its distribution in samples of n will be Poissonian; i.e. the probability (P) of finding r contaminants in a single sample of n cells is $e^{-z}z^r/r!$, and the probability of finding no contaminant at all is e^{-z} . Hence in 5 % of cases no contaminants will be observed when the expected number is three ($P=e^{-3}=0.05$). For example, even if no contaminants are found in a sample of 1000 colonies examined, there is still a 5 % chance that the degree of contamination is really as high as 0.3 %. The same considerations apply in any form of sterility testing and are discussed in more detail by Elsworth, Telling & Ford (1955).

It is therefore practically impossible to show that a culture is completely free from contaminants by a single test. Repeated tests over long periods are more significant, however, since the permanent existence of a low equilibrium

level of contaminants is improbable; foreign contaminants (or mutants) are most likely either to displace the parent culture altogether, or to disappear.

With the above reservations it can be said that over the past two years no difficulty has been found in operating the continuous culture apparatus for long periods without any detectable contamination. The longest individual run lasted 108 days and was still free from contamination when terminated voluntarily; 2–3 months is the average period for most runs. The construction of a leak-free apparatus did not prove too difficult, and contaminations experienced in the earlier stages of the work could usually be ascribed to defective initial sterilization or faulty aseptic technique during sample-taking or addition of anti-foam. If due attention be paid to these points, we see no reason why continuous cultures should not be maintained free from contamination more or less indefinitely.

So far as mutations are concerned, we have never during the longest periods of continuous operation been able to detect any organisms differing from the parent strain in microscopic or colonial morphology, biochemical reactions or growth characteristics. In view of the possibility of faster-growing though morphologically identical mutants displacing the parent strain more or less completely, we have on numerous occasions re-determined the value of the growth-rate constant μ_m at intervals during the course of a continuous culture. This can easily be done by stopping the medium flow, draining off 95 % or more of the culture (the remainder serving as inoculum), re-filling the culture vessel with fresh medium and growing up as a batch culture, the growth rate and yield constant being determined as in Fig. 4. No significant change in μ_m or Y was ever observed, even after long periods of continuous culture (cf. curves (a) and (b) of Fig. 4).

Quantitative tests of continuous culture theory. Table 1 summarizes quantitative data on steady-state bacterial and substrate concentrations at twenty-one different flow-rates. These were all obtained during a single run, over a period of 65 days continuous operation. In Fig. 6 (a)–(d) the steady-state bacterial concentration and output, substrate concentration and yield constant are plotted against flow-rate, for comparison with the results to be expected according to Monod's theory.

The theoretical curves shown were plotted from equations (9) and (10), using the values $\mu_m = 0.85 \text{ hr.}^{-1}$ and $Y = 0.53$ obtained in the batch culture experiments. Determination of K_s was more difficult. Accurate values are not readily obtained from batch culture experiments, since at the low substrate concentrations necessary, s is continually decreasing during the experiment. Theoretically, K_s is most easily determined from continuous culture experiments, for a single measurement of the steady-state substrate concentration at any dilution rate allows K_s to be calculated if μ_m is known, as is shown by re-arranging equation (9) in the form

$$K_s = \bar{s} \left(\frac{\mu_m - D}{D} \right). \quad (26)$$

In particular, when $D = \mu_m/2$, we have $\bar{s} = K_s$, and this is probably the most accurate method for determining K_s , provided the analytical method for

determining \hat{s} is sufficiently sensitive. In the present case, unfortunately, the value of K_s was so small that over most of the lower range of dilution rates the substrate concentration was too low to measure with the available methods for glycerol estimation (Table 1). K_s was therefore determined from equation (14), using experimentally determined values of μ_m and the maximum output

Table 1. *Quantitative data on the continuous culture of Aerobacter cloacae*

The apparatus and general plan of the experiment are described in the text. Each row of figures in the table refers to a different steady state. Glycerol concentration in the inflowing culture medium (s_R) was 2.5 g./l.

Flow rate (l./hr.)	Duration* of test	Bacterial concentration (g. dry wt./l.)	Output of bacteria (g./hr.)	Glycerol concentration in culture (g./l.)	Yield constant (g. bacteria g. glycerol)
f	(hr.)	\bar{x}	$f\bar{x}$	\bar{s}	$\frac{\bar{x}}{s_R - \bar{s}}$
0	(Batch culture)	1.32†	—	0	0.53†
4.6	22	1.33	6.12	<0.03‡	0.55
4.8	26	1.39	6.66	<0.03	0.57
5.0	25	1.30	6.48	<0.03	0.53
7.2	25	1.26	9.05	<0.03	0.52
8.4	14	1.25	10.47	—	—
10.0	25	1.29	12.88	<0.03	0.53
11.5	22	1.31	15.04	<0.03	0.54
11.9	24	1.27	15.08	—	—
13.8	23	1.23	16.92	—	—
13.9	34	1.22	17.0	<0.03	0.50
14.3	16	1.04	14.9	<0.03	0.43
14.5	17	1.07	15.5	—	—
15.7	25	0.96	15.1	<0.03	0.40
15.8	23	1.26	19.0	<0.03	0.52
15.9	20	0.91	14.3	<0.03	0.36
16.6	22	1.00	16.5	—	—
16.7	24	0.98	16.3	0.26	0.44
17.3	12	0.95	16.4	0.28	0.43
18.3	8	0.72	13.1	0.88	0.45
19.5	6	0.35	6.84	1.66	0.45
20.0	9	0.48	9.68	1.37	0.42
22.4	8	0.24	5.31	1.93	0.34

* The culture was allowed to stabilize for 18–24 hr. at each flow-rate before observations commenced; it was then sampled hourly during the test period. The figures for bacterial and glycerol concentrations are the means of the hourly samples.

† Means of a number of batch culture experiments.

‡ This figure represents the lowest concentration of glycerol which can be measured with accuracy by the method used.

dilution rate D_M and solving for K_s . The value obtained, which may be subject to considerable error, was $K_s = 1.35 \times 10^{-4}$ M, or 12.3 μ g. glycerol/ml. This is comparable with the values of K_s found by Monod (1942) for other carbohydrate substrates (namely 4, 2 and 20 μ g./ml. for glucose, mannitol and lactose respectively), but considerably higher than the value of 10^{-3} μ g./ml. found by Novick & Szilard (1950) with tryptophan as substrate. The above values of μ_m , K_s and Y were used in calculating the 'theoretical' curves shown in Fig. 6.

Qualitatively, the results plotted in Fig. 6 may be seen to be in general agreement with the theory. The steady-state concentration of organisms falls only very slowly as the dilution rate is increased from zero to about 0.8 hr.^{-1} (Fig. 6a); as the flow-rate is increased still further the bacterial concentration drops fairly steeply, the curve extrapolating to complete wash-out at $D = D_c = 1.2 \text{ hr.}^{-1}$ (approx.). The output of bacteria (Fig. 6b) is nearly proportional to the flow-rate up to a maximum at $D_M = 0.79 \text{ hr.}^{-1}$, thereafter falling fairly sharply to zero. The curve for steady-state glycerol concentration (Fig. 6c) should be a mirror-image of that for bacterial concentration (cf. Figs. 1

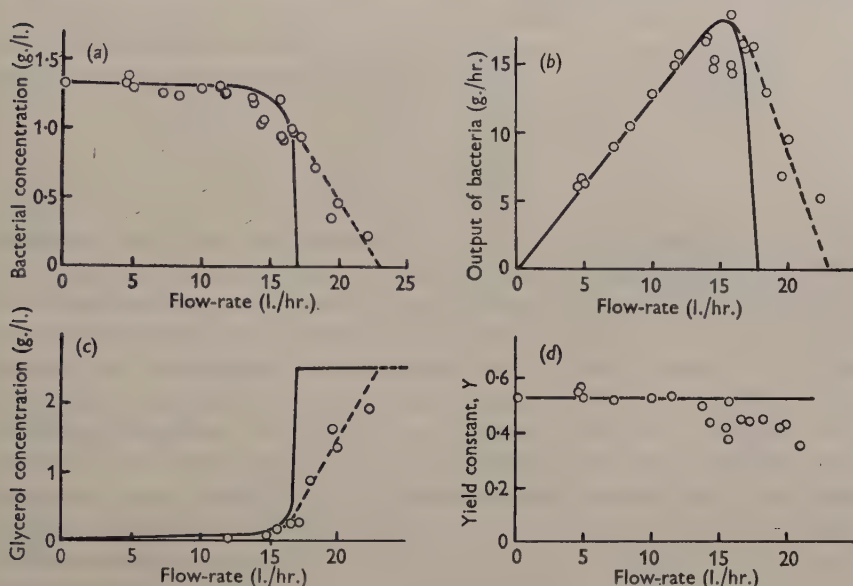


Fig. 6. Quantitative relationships in continuous culture of *Aerobacter cloacae*; comparison of experiment with theory. The data of Table 1 are plotted for comparison with the theoretical relationships calculated from equations (9) and (10). The continuous lines are the theoretical curves; the dotted lines are drawn to fit the experimental points.

and 2), and this is seen to be approximately the case; at all flow-rates up to about 16 l./hr., however, utilization of substrate was so nearly complete that the residual glycerol was too low for accurate measurement.

Quantitatively, agreement with theory is good at all the lower flow-rates (below $D = 0.8 \text{ hr.}^{-1}$), discrepancies being less than the experimental errors of bacterial dry weight and glycerol determinations. At the higher flow-rates, however, there are definite discrepancies; the curves for bacterial concentration and output and for glycerol concentration, though of the right general shape, show definite deviations from the predicted curves, bacterial concentrations being higher and substrate concentrations being lower than the values expected. In particular, the observed value of the critical dilution rate is definitely higher than that calculated from equation (12), and genuine steady-state conditions appear to have been achieved at dilution rates with $D > \mu_m$,

when theory predicts that complete 'wash-out' should occur. Another discrepancy is that the yield constant Y , which at low dilution rates has the same value as was found in batch culture experiments, shows a definite tendency to decrease at higher dilution rates (Fig. 6*d*). Possible reasons for these divergences are discussed below.

DISCUSSION

One object of the present work was to compare the results obtained during the operation of a continuous culture apparatus with those predicted by the theory given earlier. It is also relevant to compare them with the results predicted by the theories of other workers.

A common feature of all theories of continuous culture is the elementary one that net growth must be the resultant of exponential growth minus exponential 'wash-out', or re-writing equation (5):

$$\frac{d(\log_e x)}{dt} = \mu - D. \quad (27)$$

Hence when a steady state ($d(\log_e x)/dt = 0$) exists, then $\mu = D$, i.e. the specific growth rate must be equal to the dilution rate. An error made by a number of authors in mathematical discussions of continuous culture is to confuse, in the above equation, the specific growth rate μ (which varies with the substrate concentration) and the growth rate constant μ_m . Obviously, if one puts in equation (27) $\mu = \mu_m = \text{constant}$, it follows that a steady state is possible only at one particular flow-rate, when $D = \mu_m$. This is the assumption made by Golle (1953) who states that 'there is only one rate of medium flow . . . at which steady-state conditions will be maintained'. Finn & Wilson (1954) also stress this point, and the same idea appears to be implicit in the writings of Adams & Hungate (1950) and Northrop (1954). If this were so, a continuous culture would be an inherently unstable system and very difficult to operate; moreover, restriction to a single flow-rate would greatly limit its usefulness. The experimental data presented in this paper show conclusively that a continuous culture is an inherently stable system adjusting itself automatically to changes in dilution rate. Any number of steady states can be obtained at different dilution rates anywhere between zero and the critical value, as the theories of Monod (1950) and Novick & Szilard (1950) predict.

The essential features of the Monod-Novick & Szilard theories and the theoretical treatment given in this paper* is that they take into account the observed facts that bacteria can grow only at the expense of the substrate utilized, and that their specific growth rate is a function of the substrate concentration. It then becomes apparent that a continuous culture apparatus is a device for controlling growth through control of the substrate concentration; each dilution rate fixes the substrate concentration at that value which makes μ equal to D .

* We wish to emphasize that the theoretical treatment given in this paper is merely an expansion and development of those of Monod and of Novick & Szilard (particularly the former), and is based on the fundamental principles which they originated.

This important role of the substrate is not considered in the mathematical papers of Golle (1953), Finn & Wilson (1954) and the other authors mentioned above. This has led to some incorrect conclusions. For example, Golle (1953) discusses at some length the mathematics of a series of two or more culture vessels run in cascade, and concludes that there are distinct advantages in this procedure. Now it is apparent from the theory given earlier that over the useful range of flow-rates in a continuous culture, the substrate is nearly completely utilized and the issuing medium virtually exhausted; hence negligible growth could occur in any subsequent culture vessels in series with the first. Our experimental results confirm that this almost complete utilization of substrate does in fact occur.

It is to be emphasized that the theoretical treatment of continuous culture given in this paper is based on the minimum number of extremely simple postulates. It might well be objected that even under ideal conditions the growth behaviour of bacteria cannot be completely represented by such simple equations as (2), (3) and (4). Further refinement of the theory will undoubtedly become necessary as knowledge of the subject increases. The gratifying degree of agreement between experimental and predicted results shows, however, that the basic principles of the theory must be sound. Qualitatively, it provides a good general picture of the behaviour of a continuous culture, and quantitative agreement is good over most of the range. It is necessary to discuss, however, the quantitative deviations from theory found at high flow-rates, and in particular the apparent existence of steady states at dilution rates higher than the maximum specific growth rate μ_m .

According to equation (27) the specific growth rate must be equal to the dilution rate; when the latter exceeds the maximum possible growth rate, complete 'wash-out' should occur and no steady state should be possible. Since steady states were in fact found with $D > \mu_m$, this must mean that either (a) the maximum growth rate in a continuous culture is higher than in a batch culture (the value of μ_m was determined from batch culture experiments), or (b) the wash-out rate is less than would be predicted from equation (1). We believe the latter explanation to be correct.

A possible reason for an organism growing faster in continuous culture than in batch culture might be the selection of faster-growing mutants. However, as previously mentioned, no evidence for any permanent selection of this kind was found, the value of μ_m being unchanged after long periods of continuous culture. Another explanation could be based on the normal variation in the growth-rates (or generation times) of individual bacteria; in a continuous culture the faster-growing bacteria might be selected. This possibility seems to be eliminated by the work of Powell (1955), who found that there is zero correlation between the generation time of a bacterium and the generation times of the two daughter-cells into which it divides. In other words, an unusually fast-growing organism is just as likely as not to have unusually slow-growing progeny; selection would therefore not affect the mean generation time.

The alternative explanation, suggested by our colleague Mr E. O. Powell, is

that the actual wash-out rate in the culture vessel is effectively less than that given by equation (1). Though this equation has been accepted by all previous writers on the subject, it is based on the unverified assumption of 'perfect mixing' within the culture vessel. This implies that each drop of liquid entering the vessel is uniformly distributed throughout its contents in an infinitesimal time, a condition difficult to attain in practice even with vigorous stirring. Incomplete mixing means that the dilution rates in different regions of the culture vessel will not be uniform; there will be a distribution of local dilution rates about the mean or overall dilution rate $\bar{D}=f/v$. Hence when \bar{D} is greater than the critical value D_c (calculated from equation 12) there might still be regions within the culture vessel where the local dilution rate was less than D_c , and in these regions organisms will continue to be produced, so that complete wash-out will not occur. Rough calculations show that quite small deviations from perfect mixing could have surprisingly large effects, owing to the steep descent of the D against x curve in the critical region (Fig. 1). A theoretical and experimental study of this subject is being made by Mr E. O. Powell, and it will not be further discussed here, but we believe that the greater part of the deviations from theory found in our experiments can be explained in this way. This view is reinforced by the fact that in similar experiments with the same organism in a laboratory scale continuous culture apparatus fitted with a highly efficient mixing system, the deviations from theory at high flow rates are very small (Herbert, D., to be published).

Another 'apparatus effect' can produce results similar to those of imperfect mixing. During long experiments, a solid film of bacteria of considerable thickness builds up on the walls of the culture vessel above the liquid level, portions of which are continually becoming detached by splashes or condensed liquid running down the walls. This continued re-inoculation of the culture from the walls also leads to a continued production of bacteria at flow-rates which should theoretically cause complete wash-out. It is difficult to estimate the magnitude of this inoculation rate, but suppose it to be w g. bacteria/l./hr., then it can be shown that

$$\tilde{x} = \frac{w}{D - D_c}. \quad (28)$$

Hence if D is only a little above D_c , a quite small value of w can produce a quite high value of \tilde{x} .

Other imperfections in the apparatus (e.g. short-term fluctuations of flow-rate or liquid level about the mean values) can also be shown to have effects in the same direction. On the whole, it appears that most of the observed discrepancies can be attributed to 'apparatus effects' of this sort, rather than to inadequacy of the biological side of the theory. Such effects, however, cannot account for the apparent decrease in the yield constant Y at high flow-rates, and it is possible that the independence of yield constant and growth rate assumed in equation (4) may need to be modified; this point is now under investigation.

On the practical side, we consider that the results reported show that the continuous culture of bacteria on a fairly large scale is a readily feasible

proposition with apparatus of quite simple design. Complexity was deliberately avoided and the apparatus was designed for production rather than research purposes; nevertheless, the degree of control available permits of useful quantitative investigations. Results are comparable with those obtained in smaller and more complicated types of laboratory continuous culture apparatus in use in this Department. The high production rate may be emphasized; though the working capacity of the culture vessel was only 20 l., no difficulty was found in producing culture at the rate of 300 l./day and higher rates could be achieved with faster-growing organisms. The plant could be scaled up considerably without any major changes in design.

Contamination, often alleged to be a major obstacle to the operation of continuous culture on a plant scale, was not found to be a real difficulty, and runs of several months' duration are now routine. Another alleged difficulty, that of mutation, has not troubled us at all, no changes in the culture having been observed after long periods of continuous operation. In this respect our results are at variance with those of other workers (summarized by Novick, 1955) who have used continuous culture techniques as a means of studying mutation rates. It might be argued that this was due to the type of growth medium used in our experiments, since almost every mutation (of those affecting nutrition at least) would be at a disadvantage compared with the parent type. However, we have had similar experiences with other types of chemically defined and complex growth media, and it could also be argued that in much of the work done on mutations in continuous culture the conditions have been favourable for mutant survival. For a given organism and growth medium, we believe that mutants are somewhat less likely to build up in a continuous culture than in a batch culture. For mutations are rare events arising singly, and a single organism with a generation time τ has a probability of $1 - e^{-D\tau}$ of being washed out of the culture vessel before it has divided once. Hence an appreciable fraction of the mutants arising in a continuous culture will be removed before they have progeny, while in a batch culture all will remain.

In both the theoretical and experimental parts of this paper, continuous cultivation has been regarded as a process for converting substrate into bacteria—a deliberately one-sided approach. Obviously it has many other aspects; for example, as a research tool for elucidating problems of biosynthesis or as a process for the production of metabolic products. It is hoped to make these the subjects of future investigations.

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Influenza Virus Nucleic Acid: Relationship between Biological Characteristics of the Virus Particle and Properties of the Nucleic Acid

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SUMMARY: Five A strains and three B strains of influenza virus were purified and found to contain ribonucleic acid in amounts varying from 0.75 to 1.1 %. The proportion of the purine and pyrimidine derivatives in the nucleic acid of each strain was determined. When the ratio adenine + uracil : guanine + cytosine was evaluated, the following values were obtained: A strains—PR 8, 1.27 ± 0.02 ; MEL, 1.22 ± 0.01 ; WSE, 1.26 ± 0.01 ; SWINE, 1.24 ± 0.04 ; CAM, 1.28 ± 0.01 . B strains—LEE, 1.42 ± 0.04 ; MIL, 1.43 ± 0.05 ; ROB, 1.38 ± 0.01 . The nucleic acid content of PR 8 virus preparations of varying degrees of incompleteness was determined both by specific absorption at 260 m μ . and by estimation of phosphorus present in the nucleic acid extract. As measured by both methods, virus preparations of low infectivity were found to have a decreased nucleic acid content. The demonstration of specific differences in the nucleic acid of A and B strains and of the relationship between the infectivity and nucleic acid content of the virus affords strong evidence that the nucleic acid is an intrinsic part of the influenza virus particle.

There can now be little doubt that the nucleic acid present in purified preparations of influenza virus is of the ribose type. Though Knight (1947) found that a sample of nucleic acid isolated from defatted virus was degraded by crystalline ribonuclease, specific chemical evidence has been obtained only recently. As judged by absorption measurements at 260 m μ . it was shown that the nucleic acid could be quantitatively extracted from defatted virus with hot 10 % sodium chloride solutions (Ada & Perry, 1954). A sensitive test for deoxypentoses, when applied to the nucleic acid extract, gave a negative reaction (Ada & Perry, 1954), and the purine-bound pentose was later identified chromatographically as ribose (Ada & Gottschalk, 1956). On treatment with alkali at 37° for 18 hr. the isolated nucleic acid was quantitatively degraded to components which remained in solution on subsequent acidification (Ada & Perry, 1954): deoxypentose nucleic acids are not appreciably degraded under these conditions (Schmidt & Thannhauser, 1945). In preliminary experiments chromatographic analysis of the purine bases and pyrimidine nucleotides, liberated by acid hydrolysis, revealed only four components, coinciding in R_f value with those from a similarly treated sample of purified yeast ribonucleic acid (Ada & Perry, 1955*a*). Liu, Blank, Spizizen & Henle (1954) reported that a hot trichloroacetic acid extract of virus contained adenine, guanine, cytosine and uracil: no thymine was found.

The small amount (0.75-1.1 %) of ribose nucleic acid associated with influenza virus preparations of high infectivity (Ada & Perry, 1954) raises the question whether the nucleic acid is a functionally significant part of the virus

particle. There are two ways in which this might be established. The first would be by the demonstration (as in the deoxyribonucleic acid of T_{even} bacteriophages) of a constituent in the nucleic acid not present in the host cell. Almost equally cogent evidence would be provided by showing that biological differences in strains or preparations of influenza viruses were correlated with changes in the amount or constitution of the virus nucleic acid. Both approaches have been used. The evidence (Ada, unpublished) indicated that the virus RNA contains only the same nitrogenous bases as are present in yeast RNA. On the other hand, the functional significance of influenza virus nucleic acid is indicated by the two sets of observations with which the present paper is concerned: (i) that 'incomplete' virus preparations have a lower content of RNA than the standard virus; (ii) that the ribose nucleic acids from influenza A and B strains show differences in constitution according to serological type. Brief accounts of these findings were reported previously (Ada & Perry, 1955*a*, *b*).

METHODS

Strains of influenza virus. The following strains of virus were used:

A strains: PR 8 (Francis, 1934); MEL, 'Melbourne' strain (Burnet, 1935); SWINE, Shope's strain 15 of swine influenza (Shope, 1931); WSE (Smith, Andrewes & Laidlaw, 1933); CAM (Burnet & Anderson, 1947).

B strains: LEE (Francis, 1940); MIL, isolated from Melbourne epidemic (Burnet, Stone & Anderson, 1946); ROB, isolated from Melbourne epidemic (see Ledinko & Perry, 1955).

Virus fluids. Virus fluids were obtained from embryonated eggs inoculated in the allantoic cavity on the 11th day of incubation and maintained at 35°.

Standard virus. In early experiments the virus used for inoculation purposes was high titre allantoic fluid stored at -70°. Allantoic fluids were harvested 42 hr. after inoculation of 0.05 ml. of a 10^{-4} dilution of the seed virus. In later work, seed virus was passaged three times (10^{-4} dilution for 20 hr. before production of virus for the main experiment) 0.05 ml., dilution 10^{-6} ; incubation time 42 hr. In this way, inactivated virus was eliminated from the seed virus, resulting in the production of virus of higher infectivity (Horsfall, 1954).

'Incomplete' virus was grown by two or three serial passages of indiluted, infected allantoic fluid (von Magnus, 1946). Incubation times were: first (and second) passage, 16 hr.; final passage, 12 hr. (Fazekas & Graham, 1954).

Samples for haemagglutinin and infectivity estimations were taken immediately after pooling harvested fluids.

Haemagglutinin titration. Serial twofold dilutions of virus were prepared in 0.25 ml. volumes in saline (0.85 %, w/v) and 0.25 ml. of a 1 % suspension of fowl red blood cells was added to each tube. The standard + pattern of cell agglutination was taken as end-point and was read after the cells had settled for 45 min. at room temperature. The reciprocal of the end-point dilution was taken as the titre; all titrations were carried out in duplicate.

Infectivity titration. Serial tenfold dilutions of virus fluids were prepared in ice-cold saline containing 10 % normal horse serum, and 0.05 ml. of each

dilution was inoculated into the allantoic cavity into each of twelve 11-day embryonated eggs. After 3 days of incubation at 35°, the eggs were tested for the presence of virus haemagglutinin in the allantoic fluid. ID₅₀ end-points were calculated by the method of Reed & Muench (1938). For comparative purposes, the ratio I/A is used, where $I = \text{ID}_{50}$ and $A = \text{haemagglutinin titre}$.

Purification of virus. All virus preparations were purified according to the same procedure, comprising specific adsorption to and elution from human red blood cells followed by differential centrifugation. Two modifications were introduced in the method published earlier (Ada & Perry, 1954): (i) to facilitate elution of the adsorbed virus from the erythrocyte surface, approximately 20,000 units of a receptor-destroying enzyme preparation (Ada & French, 1950) were added to the eluting fluid; (ii) conditions of centrifugation in the centrifugation cycles were changed to the following: sedimentation, 38 000 g, 30 min.; clarification, 7000 g, 10 min. The yield of purified virus was expressed as the percentage recovery of haemagglutinin.

Purity of virus. The purity of the preparations was judged by examination in the ultracentrifuge and electron microscope (see the following paper, Pye, Holden & Donald, 1956) and assessed by determination of the ratio, haemagglutinin units/mg. dry weight.

Extraction of nucleic acid from virus. The purified virus was precipitated by the addition of trichloroacetic acid, dialysed against distilled water and dried from the frozen state *in vacuo*. After defatting the dried virus with chloroform + methanol (2 : 1) the nucleic acid was quantitatively extracted from the defatted virus with hot 10% (w/v) NaCl solution (Ada & Perry, 1954). Addition of 2 vol. of ethanol to the extract precipitated the nucleic acid which was washed with 66% ethanol, 95% (v/v) ethanol in water, and finally with freshly distilled ethyl ether.

Estimation of virus nucleic acid (VNA). The nucleic acid content of the 10% sodium chloride extract was estimated in two ways. The specific absorption at 260 m μ . was determined. The formula, $\mathcal{E}_p = 30.98 E/cl$ where \mathcal{E}_p = atomic extinction coefficient at 260 m μ . with respect to phosphorus, E = optical density, c = concentration of phosphorus in g./l., and l = thickness of absorbing layer (Chargaff & Zamenhof, 1948) was used to determine spectrophotometrically the nucleic acid content. The value $\mathcal{E}_p = 9200$ was experimentally determined on VNA reprecipitated as above and dissolved in water. The factors 9.7 for A strains and 9.6 for B strains, evaluated from the known proportions of bases (Ada & Perry, 1955*b*), were applied to convert the phosphorus value so obtained to amount of VNA. In this calculation of \mathcal{E}_p , no account is taken of possible effects of heating RNA and subsequently measuring the optical density in 10% NaCl. To investigate this, a sample of RNA was prepared from guinea-pig liver by the method of Kay & Dounce (1953). The purified preparation contained 8.4% phosphorus and had an \mathcal{E}_p (in water) of 9300. Approximately 1.5 mg. was dissolved in 10 ml. water; one of two 1 ml. samples was added to 1 ml. water and the other to 1 ml. 20% NaCl solution. The optical density reading in 10% NaCl was 3% lower than that in water (see Shack,

Jenkins & Thompson, 1953). Both solutions, in sealed tubes, were placed in a boiling water-bath for 20 min., cooled and the optical density measured. An increase of *c.* 1.5 % occurred in both cases. Thus the depression of the optical density in 10 % NaCl is largely cancelled by the increase following heating. In view of this result, the value $\epsilon_p = 9200$ for VNA as determined above was used uncorrected. A direct determination of phosphorus in the sodium chloride extract and conversion of this value to nucleic acid amount using the appropriate factor given above was also made. Phosphorus determinations were carried out as previously described (Ada & Perry, 1954).

Yeast nucleic acid (YNA). A commercial preparation of yeast ribose nucleic acid was purified according to the procedure of Smith & Markham (1950).

Paper chromatography

Hydrolysis of nucleic acid. 50–250 μ g. nucleic acid (purified YNA or reprecipitated VNA) was hydrolysed with 50 μ l. N-HCl (100°, 60 min.) in a sealed tube. 40–50 μ l. of the hydrolysate was applied to paper. Whatman no. 1 paper, washed according to the method of Hanes, Hird & Isherwood (1952), was used in ascending chromatographic analysis. The width of the paper strips varied from 0.7 to 2.0 cm. according to the amount of nucleic acid (as hydrolysate) applied. The solvent used was isopropanol + conc. HCl + water in the proportions 65 : 18 : 17 (Wyatt, 1951). The solvent front moved approximately 26 cm. in 18 hr.

Detection and estimation of the separated purine and pyrimidine derivatives

The paper was air-dried, the components located by their absorption in the ultraviolet light, and in each case a photographic record was made (Markham & Smith, 1951). The ultraviolet-absorbing spots were cut out (with corresponding areas from a blank strip) and the purine and pyrimidine derivatives eluted for 18 hr. at room temperature with 10 ml. 0.1 N-HCl (Smith & Markham, 1950). The optical density of the eluates contained in 4 cm. cuvettes was read at the wavelength of maximal absorption for each derivative, and the concentration determined by applying the appropriate extinction coefficient (Markham & Smith, 1951).

With every chromatographic analysis of a VNA hydrolysate, a YNA hydrolysate was run as a control. Unless the values obtained for the proportions of bases (expressed as the ratio adenine + uracil : guanine + cytosine) agreed within ± 2.5 % of a mean value, 0.90, obtained in a preliminary series of experiments, the result of the accompanying VNA analysis was discarded. Analytical results for the distribution of bases in incomplete virus may not have this accuracy because of the small amounts (less than 70 μ g.) of nucleic acid which were examined. In several experiments, the recovery of phosphorus (evaluated from base recoveries, assuming equimolar amount of bases and phosphorus) from the chromatogram was compared with the known amount of phosphorus applied. Recoveries between 100 and 103 % were obtained with both VNA and YNA.

RESULTS

Infectivity of virus preparations. The production of standard virus (PR 8 strain) from glycerolated seed resulted in preparations with an average I/A value of 5.7 ± 0.4 . In later experiments in which the virus to be used as seed was previously passaged several times at high dilution, the average I/A value of the unpurified virus (PR 8) was 6.0 ± 0.3 . Other strains of virus, produced by a single passage from dilute inoculum, gave the following average I/A values: strains MEL, 6.1; CAM, 6.0; WSE, 5.6; SWINE, 5.9; strains ROB, 6.5; MIL, 5.6; LEE, 6.0. The results given by preparations of incomplete virus (PR 8) were as follows: two passages of undiluted inoculum, $I/A = 4.3 \pm 0.4$; three passages of undiluted inoculum, $I/A = 3.8 \pm 0.4$.

Yield of purified virus. Similar yields were obtained with all the A strains used, irrespective of the infectivity of the starting material. Two B strains, LEE and MIL, were recovered in lowest amounts. Few losses occurred during the purification of the B strain ROB as indicated by the high recovery of haemagglutinin. The average yields, together with the ranges, are given in Table 1.

Table 1. *The yield, haemagglutinating activity (haemagglutinin units/mg. dry weight) and nucleic acid content of some A and B strains of influenza virus*

Virus*		No. of experiments	Yield (%)	Haemagglutinin units per mg. dry weight ($\times 10^{-4}$)	Nucleic acid content† (%)
A	PR 8	9	$47 \pm 16^\ddagger$ (27-73)	9.2 ± 1.6 (6.9-11.2)	See Figs. 1a and b
	PR 8§	5	47 ± 13 (40-66)	10.7 ± 1.5 (10.0-12.1)	
	PR 8	5	36 ± 14 (21-57)	7.2 ± 0.8 (6.2-8.0)	
	MEL	2	38 (30-46)	9.4 (7.2-11.6)	0.84 (0.85-0.82)
	CAM	2	33 (22-43)	8.1 (6.4-9.7)	0.88 (0.94-0.81)
	WSE	2	41 (32-50)	6.6 (6.4-6.9)	0.85 (0.81-0.89)
	SWINE	2	37 (26-48)	7.5 (7.0-8.1)	0.79 (0.75-0.83)
B	ROB	2	80 (70-90)	9.7 (8.1-11.3)	0.94 (0.87-1.01)
	MIL	3	18 (15-25)	2.9 (2.3-3.4)	0.80 (0.75-0.83)
	LEE	2	24 (20-28)	3.5 (2.8-4.3)	0.94 (0.94-0.94)

* With two exceptions, viruses grown by single passage from dilute inoculum.

† Estimated by absorption in the ultraviolet.

$$\ddagger \text{ S.D.} = \sqrt{\frac{\sum(\bar{x} - x)^2}{n-1}}.$$

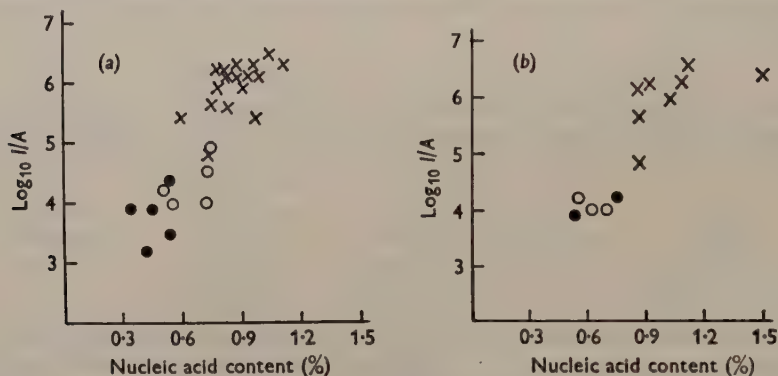
§ Two serial passages of undiluted inoculum.

|| Three serial passages of undiluted inoculum.

Purity of virus preparations. A comparison of the ratio, haemagglutinin units/mg. dry weight, indicated that the values of all the A strains and the B strain ROB fell within the range $6-12 \times 10^4$. The two B strains, LEE and MIL, consistently gave values of a lower order ($2.3-4.3 \times 10^4$) (Table 1).

Nucleic acid content. In earlier work the nucleic acid content of the A strain PR 8 (standard preparations) was found to be *c.* 0.8% as estimated by ultra-violet absorption (Ada & Perry, 1954). It is clear from Table 1 that all of the other strains of virus tested contain similar amounts of nucleic acid.

Relationship between the infectivity and nucleic acid content of virus. The nucleic acid content of PR 8 virus preparations, whose I/A values ranged from 6.5 to 3.2, was determined both by specific absorption at 260 $m\mu$. and by estimation of phosphorus present in the nucleic acid extract. The values obtained are plotted in Fig. 1 *a, b*, the nucleic acid content being expressed as %/mg. dry weight of virus. It is evident that, as measured by both methods, the virus preparations of low infectivity had a decreased nucleic acid content.



Figs. 1 *a* and *b*. Relationship between the infectivity/haemagglutinin ratio and the nucleic acid content of influenza virus. x = single passage (0.05 ml., ID $50 = 10^{-4} - 10^{-8}$, 42 hr.); o = double passage (each 1.0 ml. undiluted fluid); ● = triple passage (each 1.0 ml. undiluted fluid). Fig. 1 *a*. Nucleic acid content estimated by absorption at 260 $m\mu$. of nucleic acid extract. Fig. 1 *b*. Nucleic acid content estimated from phosphorus content of nucleic acid extract.

Proportions of nitrogenous bases of the nucleic acid isolated from virus preparations (PR 8) of varying infectivity. It was thought possible that some indication of the specificity of the missing components in incomplete virus might be obtained by a comparison of the base proportion in the nucleic acid isolated from incomplete virus with the values of the nucleic acid from standard virus. In each case, the nucleic acid was hydrolysed, chromatographed and the amount of purine and pyrimidine derivatives estimated. The results (Table 2) indicate that in two preparations of low infectivity ($I/A = 4.5, 3.9$), the values were within experimental error of those found in preparations of high infectivity.

Specific differences in the nucleic acids from A and B strains of virus. Five A strain and three B strain viruses were analysed for their base proportions as described above (Table 3). It can be seen that, in general, the guanine content

of the B strains is lower than that of the A strains; the uracil content of the A strains is lower than that of the B strains; and both strains have similar amounts of cytosine. Comparison of the values of the ratio adenine + uracil : guanine + cytosine indicates that an A strain virus can be sharply differentiated from a B strain virus. The difference between the means of any pair of A and B viruses is significant (t test, $P < 0.05$). Differences of the mean values within each category are not significant ($P > 0.1$).

Table 2. *Proportions of bases in the nucleic acid of preparations of influenza virus (PR8) having varying infectivity/haemagglutinin ratios*

Virus PR 8 (A)	I/A	No. of experi- ments	Ratio of bases referred to adenine				Ratio: adenine + uracil guanine + cytosine
			Adenine	Guanine	Cytosine	Uracil	
	6.5-5.6	5	10	8.7 \pm 0.2	10.4 \pm 0.5	14.2 \pm 0.13	1.27 \pm 0.02
	4.5	1	10	8.5	10.2	13.7	1.27
	3.9	1	10	8.8	10.6	14.0	1.25

Table 3. *Proportions of bases in the nucleic acids of A and B strains of influenza virus*

Virus strain		No. of experi- ments	Ratio of bases referred to adenine				Ratio: adenine + uracil guanine + cytosine
			Adenine	Guanine	Cytosine	Uracil	
A	PR 8	5	10	8.7 \pm 0.2	10.4 \pm 0.5	14.2 \pm 0.13	1.27 \pm 0.02
	MEL	2	10	8.3, 8.8	10.7, 11.3	13.3, 14.5	1.22 \pm 0.01
	WSE	2	10	8.9, 8.9	10.8, 10.5	15.0, 14.3	1.26 \pm 0.01
	SWINE	2	10	9.1, 8.8	10.9, 10.6	14.1, 14.5	1.24 \pm 0.04
	CAM	2	10	8.5, 8.5	10.9, 10.7	14.7, 14.8	1.28 \pm 0.01
B	LEE	4	10	8.0 \pm 0.5	10.0 \pm 0.3	15.5 \pm 0.5	1.42 \pm 0.04
	MIL	3	10	7.7 \pm 0.2	10.4 \pm 0.3	15.8 \pm 0.2	1.43 \pm 0.05
	ROB	2	10	8.4, 8.2	10.4, 10.4	15.7, 15.9	1.38 \pm 0.01

DISCUSSION

A comparison of the results of chemical analyses carried out on different virus preparations will be valid only if each substrain or type can be obtained in a 'pure' state. In the present investigation, the degree of purification of each preparation was estimated by determination of the ratio haemagglutinin units/mg. dried weight for each purified preparation and by examination of the purified virus in the ultracentrifuge and less frequently by the electron microscope (see the following paper, Pye, Holden & Donald, 1956). By the first criterion, the figures in Table 1 suggest that all the A strain viruses had been purified to an equal extent. The ultracentrifuge patterns of the individual virus preparation generally revealed a single boundary; in some cases, a small amount of a more slowly sedimenting component was present. As the surface characteristics of incomplete virus are indistinguishable from those of standard virus (von Magnus, 1954), the similar haemagglutinating activity of the purified preparations of incomplete and standard virus in the present investigation strongly suggests that a comparable degree of purification had been achieved

in each case. Comparison of the ultracentrifugal diagrams in this series might not furnish a clear indication of purity because of the more complex pattern frequently given by incomplete virus. While the recently isolated B strain ROB had a haemagglutinating activity similar to those of A strains, the low values of the well-established B viruses, MIL and LEE may cast some doubt on the purity of these preparations. However, the consistently low values of MIL and LEE, when considered together with the ultracentrifugal evidence of a single sedimenting boundary, favour the concept that the low haemagglutinating activity is an intrinsic property of the virus particle. Preparations of virus examined by the electron microscope showed no appreciable contamination with obviously non-viral material. The available evidence suggests therefore that the purified virus preparations which were chemically analysed did not contain significant amounts of extraneous material.

The two species of influenza virus A and B used in this study are differentiated primarily by their serological behaviour. The clearest difference is in terms of the soluble complement-fixing antigen (C.F.A.) which is group specific, all A strains having a C.F.A. sharply distinct from that produced in the course of infection with a B strain. The antigens of the virus surface are more complex and the A strains in particular show very wide serological differences. In general, however, there is some cross-reaction between all A strains, while the antigenic diversity in the B strains is considerably less. There are other reasons too for thinking that the difference between the A and B species is a real one, notably the failure to obtain any evidence of recombination (Perry & Burnet, unpublished). The results of the chemical analyses are in broad agreement with the biological findings. There was a consistent and reproducible difference between the values of the ratio adenine + uracil : guanine + cytosine of the five A and three B strains tested. On the other hand, in no case were the differences between mean values within either the A or the B strains significant. If fine differences between the values of this ratio do exist within each species, it appears unlikely that the present technique, when applied to very small amounts of nucleic acid, would detect them.

With both plant and bacterial viruses examples have been found where infection is associated with the formation of particles which, though closely related (either serologically, chemically or morphologically) to the corresponding virus particle, are non-infectious. In each of the instances which have been adequately studied, the non-infectious component(s) contains little or none of the nucleic acid associated with the infectious virus particle (Markham, 1951; Jeener & Lemoine, 1953; Rich, Dunitz & Newmark, 1955; see Epstein, 1953).

A more complex situation is found with the influenza virus. The conditions giving rise to 'incomplete' virus, i.e. virus in which infectivity titrations give lower values than would be expected from haemagglutinin titres or particle counts by electron microscopy, have been discussed by numerous workers (von Magnus, 1954; Fazekas & Graham, 1954; Finter, Liu & Henle, 1955; Burnet, 1955). Here it need only be noted that exposure at 35° will also lower the infectivity titre without changing haemagglutinating activity. For this

reason precautions were taken to ensure that the virus did not remain long in the egg after its production. It can be assumed that the virus preparations used contained only a small proportion of thermally degraded virus. Even so there was considerable variation in the infectivity of virus preparations of similar nucleic acid content and in the nucleic acid content of virus preparations of similar infectivity. Though the same trend is shown independently of the method of nucleic acid determination, the estimates given by phosphorus analyses are higher than those evaluated from ultraviolet absorption measurements, due probably to the presence in the salt extract of small amounts of other organic phosphates. Clearly there is no simple proportionality between infectivity and nucleic acid content; a ninety-nine-fold decrease in infectivity was accompanied by approximately a twofold decrease in nucleic acid content. It should be mentioned that in a more limited series of experiments, it was found (Donald & Ada, unpublished) that essentially the same relationship was present when the I/A ratio was plotted against the nucleic acid content calculated on a virus particle basis. It may well be that only virus particles with a nucleic acid content above a certain value can induce continuing infection. With decrease in the amount of nucleic acid in the parent particle there will be a progressively greater decrease in the proportion of infectious progeny produced and at a certain value of nucleic acid amount, only non-infectious haemagglutinin will be produced. Such an interpretation is consistent with the concept of intermediate grades of incompleteness (Burnet, Lind & Stevens, 1955), based on the observation that incomplete virus (WS strain) produced a much higher yield of haemagglutinin than would be expected from its infectivity titre, as determined by limit dilution in the embryonated egg. Until much more is known of the process of virus replication, it would be premature to attempt a more detailed interpretation of these relationships. The fact that the proportion of bases is not significantly different in standard and incomplete virus perhaps suggests that the decrease in nucleic acid content is determined by some random process but by no means excludes the possibility that a specific moiety of the nucleic acid is concerned in determining infectivity.

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Sedimentation Behaviour and Electron Microscopic Examination of Purified Influenza Virus

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Purified preparations of influenza virus were examined in the ultracentrifuge and electron microscope principally to assess the degree of purification achieved. As indicated in the main paper (Ada & Perry, 1956) the same purification procedure was used for each batch of virus.

METHODS

Ultracentrifugation. The virus was examined in 0.85 % NaCl solution (w/v) at a concentration varying between 0.2 and 0.4 % (w/v). The ultracentrifuge was air-driven of the Beams-Pickels type. The speed of the rotor was 185 r.p.s. and the temperature *c.* 20°. The sedimentation was observed with a schlieren optical system having an inclined second slit. The light was of 5461 Å. from a mercury arc. Viscosity measurements were carried out in an Ostwald viscometer at 25°.

Electron microscopy. A Siemens microscope, type UM 100, was used. The specimens to be examined were dialysed at 0° against 0.8 % (w/v) ammonium carbonate solution, sprayed on to nitrocellulose films at a pressure of 10 lb./sq.in., air dried and shadowed with gold manganin.

RESULTS

Sedimentation behaviour. Most samples of standard virus showed a single peak of $S_{20} = 620 - 800 \times 10^{-13}$. In some cases, a small amount (< 20 %) of a slower component ($S_{20} = 350 - 500 \times 10^{-13}$) was present. The sedimentation constant was independent of concentration (within experimental error) over the range studied (0.2-0.4 %). Incomplete virus, PR8 strain, whether made by two or three passages of undiluted inoculum, consistently showed decreased sedimentation constants, varying between 400 and 550×10^{-13} . Incomplete virus was usually more heterogeneous than standard virus. Relative viscosities of the virus solutions were of a similar order to those found by Lauffer & Stanley (1944), who investigated material purified by extensive differential centrifugation.

In Pl. 1, fig. 1, are shown sedimentation diagrams of purified preparations of several A and B strains and of incomplete influenza virus.

Electron microscopy. Pl. 1, figs. 2-4, show typical fields of purified preparations of standard PR8, standard LEE and incomplete PR8 respectively.

CONCLUSIONS

The values for the sedimentation constants for both standard and incomplete virus preparations are similar to those found by earlier workers (Lauffer & Stanley, 1944; Gard, von Magnus, Svedmyr & Birch-Andersen, 1952). The frequent presence of small amounts of a more slowly sedimenting component in purified preparations of standard virus has also been previously discussed and may represent small amounts of incomplete virus (Gard *et al.* 1952). Electron micrographs show that purified preparations of standard virus exhibit a range of particle size. It is not surprising, therefore, that the sedimentation boundaries are more spread than can be explained by diffusion. The sedimentation patterns and the electron micrographs suggest that incomplete virus has a greater range of particle size than has standard virus (see also Birch-Andersen & Svedmyr, quoted by Uhler & Gard, 1954). Since the same conditions of purification were used for all batches of virus, the fact that the sedimentation diagrams of purified preparations of standard and incomplete virus contain no major component common to both suggests that most of the extraneous material has been eliminated. The absence of appreciable amounts of obviously non-viral material in the electron micrographs, together with the finding (to be published) that all the particles considered to be virus may be adsorbed on to red cells, provides the strongest evidence that a high degree of purity has been achieved.

The preliminary examination of purified viruses reported above has led to the initiation of more comprehensive study of the physical properties of standard and incomplete viruses. The results will be published at a later date.

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EXPLANATION OF PLATE

Fig. 1. Sedimentation patterns of purified influenza virus. Direction of sedimentation, right to left. Speed of rotor, 185 r.p.s. Infectivity/haemagglutinin (I/A) values and approximate sedimentation times as indicated below:

- (a) PR 8 (A) standard preparation, $I/A = 6.3$; 28 min.; $S_{20} = 660S$.
- (b) MEL (A) standard preparation, $I/A = 6.2$; 34 min.; $S_{20} = 750S$.
- (c) CAM (A) standard preparation, $I/A = 6.6$; 38 min.; $S_{20} = 620S$.
- (d) LEE (B) standard preparation, $I/A = 5.6$; 35 min.; $S_{20} = 700S$.
- (e) MIL (B) standard preparation, $I/A = 5.5$; 34 min.; $S_{20} = 640S$.

(f) ROB (B) standard preparation, $I/A=6.2$; 27 min.; $S_{20}=800 S$.

(g) PR 8 (A) incomplete preparation (two passages) $I/A=4.5$; 37 min.; $S_{20}=550 S$.

(h) PR 8 (A) incomplete preparation (two passages) $I/A=4.9$; 44 min.; $S_{20}=510 S$.

(i) PR 8 (A) incomplete preparation (three passages) $I/A=4.4$; 37 min.; $S_{20}=490 S$.

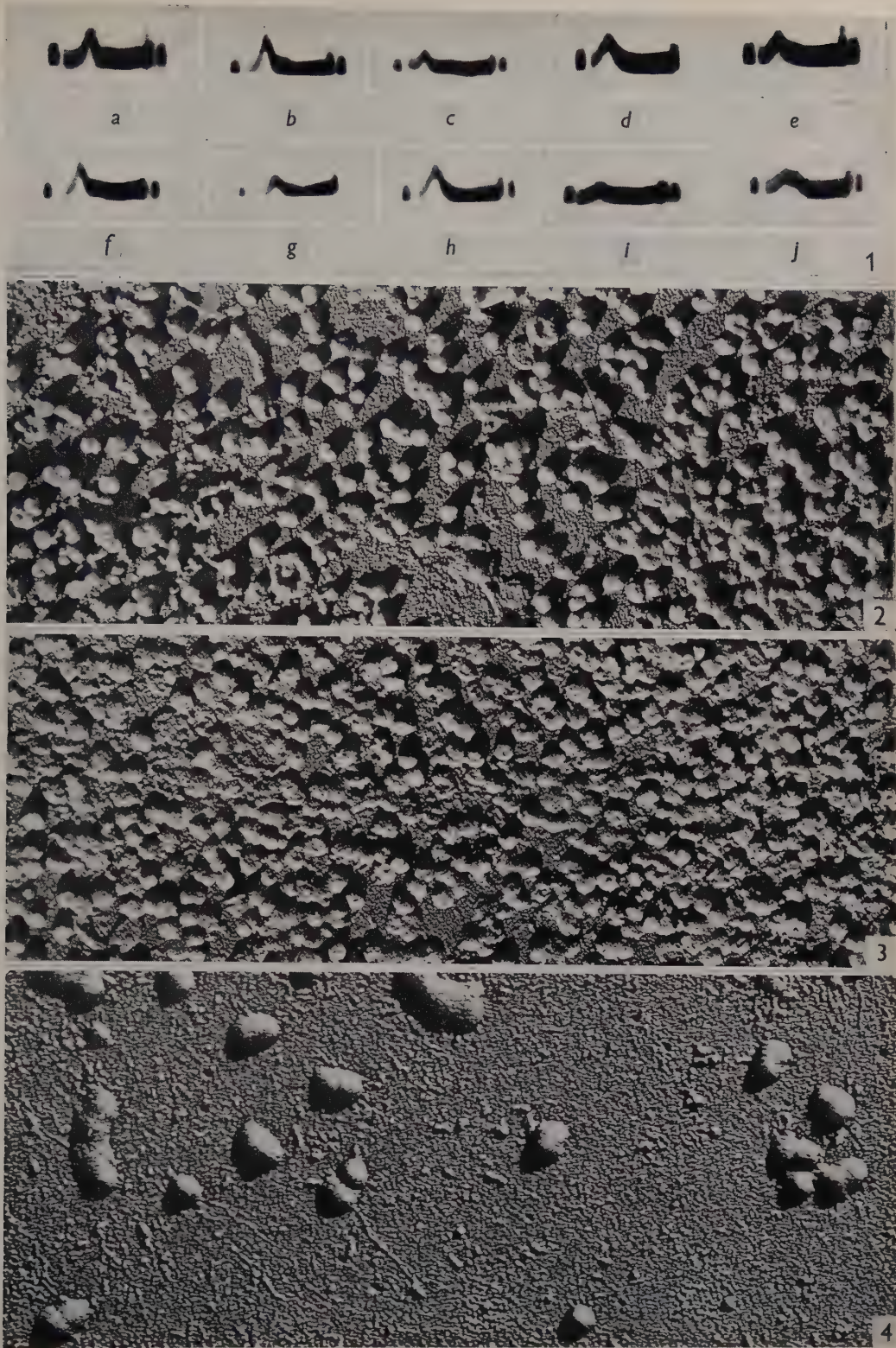
(j) PR 8 (A) incomplete preparation (three passages) $I/A=3.5$; 38 min.; $S_{20}=400, 500 S$.

Fig. 2. Electron micrograph of standard PR 8. Magnification, 30,000.

Fig. 3. Electron micrograph of standard LEE. Magnification, 30,000.

Fig. 4. Electron micrograph of incomplete PR 8. Magnification, 30,000.

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J. PYE, H. F. HOLDEN AND H. B. DONALD—EXAMINATION OF PURIFIED INFLUENZA VIRUS.
PLATE 1

(Facing p. 636)

WILLIAMSON, G. M. & WHITE F. (1956). *J. gen. Microbiol.* **14**, 637-642

Dihydrostreptomycin and Anaerobiosis—Comparison with Other Antibiotics and its Selectivity with regard to Obligate Anaerobes

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SUMMARY: The sensitivity to various antibiotics of certain facultatively anaerobic bacteria when growing under aerobic or anaerobic conditions was compared. The antibiotics tested were: dihydrostreptomycin, penicillin, chloramphenicol, erythromycin, carbomycin, chlortetracycline, oxytetracycline, tetracycline. Dihydrostreptomycin was unique amongst the antibiotics tested in being less active against facultative anaerobes when they were growing anaerobically than when growing aerobically. Dihydrostreptomycin was relatively inactive against *Clostridium welchii* and *C. novyi*, and its action was governed by the inoculum size. Dihydrostreptomycin is not a useful selective agent for the isolation of *C. welchii*.

It was observed during routine sensitivity examinations by the ditch-plate technique that streptomycin appeared to have less effect on certain sensitive facultative anaerobes when they were growing anaerobically than upon the same organisms when growing aerobically. There have been several similar observations (Bondi, Dietz & Spaulding, 1946; Geiger, Green & Waksman, 1946; May, Voureka & Fleming, 1947; Grumbach, 1950; Lightbown, 1954). These observations have led to the assumption that streptomycin or dihydrostreptomycin interfere with the oxidative mechanisms of bacterial metabolism. As far as is known, penicillin does not possess this property, but there is little information about other antibiotics (Stokes, 1955). We thought it of interest to compare the effect of penicillin and other antibiotics on facultative anaerobes when growing aerobically or anaerobically.

METHODS

Organisms. With the exception of Friedländer's bacillus (*Klebsiella pneumoniae* strain 41; May *et al.* 1947) and *Staphylococcus aureus* Oxford strain, the organisms used (*Neisseria meningitidis*, *Haemophilus influenzae* type b, *Escherichia coli*, *Aerobacter aerogenes*, *Streptococcus pyogenes*, *Diplococcus pneumoniae*, *Clostridium welchii* and *C. novyi*) were all stock laboratory strains; all were typical by routine laboratory tests. In experiments to test the efficiency of dihydrostreptomycin as a selective agent for the isolation of obligate anaerobes, in particular *C. welchii*, samples of faeces sent to the laboratory for routine examination were used.

Media. Heated blood (10 %, v/v) agar (pH 7·8; chocolate agar) was used as it satisfied the needs of the most fastidious members of the collection. When dihydrostreptomycin was tested as a selective agent for obligate anaerobes,

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the medium was Robertson's cooked meat medium, either alone or in conjunction with fresh blood (10 %, v/v) agar and sterilized milk.

Antibiotics. Dihydrostreptomycin, penicillin, chloramphenicol, erythromycin, carbomycin, chlortetracycline, oxytetracycline and tetracycline were examined. The dihydrostreptomycin sulphate (Glaxo) was dispensed in sterile distilled water in amounts necessary to give the required concentration when 1.0 ml. of the antibiotic solution was added to the suspending medium. The other antibiotics were made up in sterile distilled water to give penicillin 100 i.u./ml., and the remainder 100 μ g./ml.; these solutions were further diluted with nutrient agar, when required, to give a final concentration of 20 i.u./ml., and 20 μ g./ml. respectively.

Comparison of the activity of dihydrostreptomycin with other antibiotics

Heated blood agar (25 ml.) was poured into Petri dishes of 9 cm. diameter, cooled and dried; a ditch was then cut on one side. Eight groups of six ditch plates were used and the ditches in each group filled with nutrient agar, at 45°, containing either penicillin 20 i.u./ml. or 20 μ g./ml. of one of the other antibiotics under examination. One standard loopful of an 18 hr. culture of the organism under test grown in suitable fluid medium (diluted $1/10^4$ in nutrient broth in the case of the more vigorously growing organisms, e.g. *Escherichia coli* and $1/10^2$ in the case of the less vigorous, e.g. *Streptococcus pyogenes*) was streaked across the dry surface of the plate, beginning from the edge of the ditch. This procedure ensured that the heaviest inoculum was the first to be exposed to the diffusing antibiotic. Each group of six plates was divided into two sets of three. One set was incubated aerobically and the other anaerobically in a McIntosh and Fildes anaerobic jar for 48 hr.

Examination of the action of dihydrostreptomycin on Clostridium welchii

As previously, triplicate heated blood agar ditch plates were prepared containing 1000, 500, 200, 100 or 50 μ g. dihydrostreptomycin/ml. nutrient agar in the ditch. $1/10^1$ to $1/10^8$ dilutions of an 18 hr. broth culture of *Clostridium welchii* were prepared and one standard loopful of the undiluted culture and of each dilution streaked across the plate. Parallel experiments were carried out in Robertson's cooked meat medium. Series of tubes containing 1000, 500, 100, 50 and 25 μ g. dihydrostreptomycin/ml. medium were inoculated with 0.034 ml. of parent culture and of the $1/10^2$, $1/10^4$, $1/10^6$ and $1/10^8$ dilutions used in the experiments on solid media. The plates were examined after 48 hr. of anaerobic incubation and cultures in fluid media after 24 hr. and 5 days of incubation.

*A possible selective medium containing dihydrostreptomycin
for the isolation of Clostridium welchii*

Since obligate anaerobes are not inhibited by dihydrostreptomycin, its use as a selective agent for the isolation of *Clostridium welchii* was investigated. Tubes of cooked meat medium containing decreasing concentrations of dihydrostreptomycin were inoculated with a sample of faeces. A conventional

test for the isolation of anaerobes was set up in parallel: a tube of cooked meat medium was inoculated and then heated at 65° for 30 min. (Mackie & McCartney, 1953). After overnight incubation in air each cooked meat broth culture was subcultured to a fresh-blood agar plate. After further anaerobic incubation for 18–24 hr. these plates were examined for the presence of *C. welchii*. Typical colonies were examined microscopically and for 'stormy clot' fermentation in sterilized milk.

RESULTS

Comparison of the activity of dihydrostreptomycin with other antibiotics

The activity of the antibiotic under investigation toward a number of representative facultative anaerobes growing under aerobic and anaerobic conditions is recorded in Table 1. As controls, a strict aerobe (*Neisseria meningitidis*) and two obligate anaerobes (*Clostridium welchii* and *C. novyi*) were included. From this survey it is seen that dihydrostreptomycin was unique among the antibiotics tested in being more effective against facultative anaerobes when

Table 1. *Inhibition of growth of a number of anaerobes (facultative and obligate) separately exposed to eight different antibiotics, under aerobic or anaerobic conditions*

Organism	Conditions of growth	Linear extent (mm.) of complete inhibition of growth measured from ditch containing antibiotic							
		Dihydrostreptomycin, 20 µg./ml.	Penicillin, 20 i.u./ml.	Chloramphenicol, 20 µg./ml.	Erythromycin, 20 µg./ml.	Carbonynein, 20 µg./ml.	Chlortetracycline, 20 µg./ml.	Oxytetracycline, 20 µg./ml.	Tetracycline, 20 µg./ml.
<i>Neisseria meningitidis</i>	Aerobic	8.0	26.0	15.0	16.0	5.0	10.0	15.5	5.0
<i>Haemophilus influenzae</i> type b	Aerobic	10.0	15.5	17.0	14.0	5.0	8.0	11.0	2.0
	Anaerobic	6.0	18.0	19.0	11.0	5.0	8.0	12.0	3.0
<i>Staphylococcus aureus</i>	Aerobic	8.0	22.0	11.0	15.5	9.5	10.0	12.0	6.0
	Anaerobic	3.0	24.0	12.0	17.0	11.0	11.5	13.0	6.0
<i>Klebsiella pneumoniae</i> strain 41	Aerobic	7.0	0.0	8.0	2.0	0.0	3.5	8.0	0.0
	Anaerobic	4.0	0.0	9.0	2.0	0.0	6.0	9.5	0.0
<i>Escherichia coli</i>	Aerobic	7.0	5.0	6.0	1.0	0.0	3.5	10.0	0.0
	Anaerobic	3.0	5.5	10.0	1.0	0.0	5.5	11.0	0.0
<i>Aerobacter aerogenes</i>	Aerobic	5.0	0.0	6.5	1.0	0.0	3.0	7.5	0.0
	Anaerobic	3.0	0.0	10.0	1.5	0.0	5.5	9.0	0.0
<i>Streptococcus pyogenes</i>	Aerobic	3.0	22.5	9.0	15.5	11.5	10.5	10.0	0.0
	Anaerobic	1.0	25.5	12.0	17.5	12.0	10.0	10.0	0.0
<i>Diplococcus pneumoniae</i>	Aerobic	3.0	24.0	14.0	22.0	13.0	10.5	15.0	5.0
	Anaerobic	1.0	27.0	15.0	22.0	14.0	12.5	16.0	6.0
<i>Clostridium welchii</i>	Anaerobic	0.0	19.0	11.0	15.0	10.0	13.5	14.0	12.0
<i>C. novyi</i>	Anaerobic	0.0	23.0	15.0	17.0	11.0	16.0	16.0	12.0

Each figure is the average of the results of at least three experiments.

they were growing aerobically than against the same organisms when growing anaerobically. This is the more significant when it is realized that facultative anaerobes grow at a slower rate under anaerobic as compared with aerobic conditions (Monod, 1942). No doubt the slower rate of growth of facultative anaerobes when growing anaerobically contributes to their greater sensitivity to the other antibiotics, tested under anaerobic as compared to aerobic conditions. The resistance to dihydrostreptomycin of the two species of obligate anaerobes used as controls, though not absolute, was found experimentally to be well beyond the limit at which any organism is regarded as sensitive to the antibiotics tested. Indeed, in the case of *C. welchii* an inoculum of one organism resisted the activity of dihydrostreptomycin 50 $\mu\text{g./ml.}$ (Table 3). Taken together these observations confirm previous reports on the mode of action of streptomycin and dihydrostreptomycin under anaerobic conditions (Robinson, Smith & Graessle, 1944; Bondi *et al.* 1946; Geiger *et al.* 1946; May *et al.* 1947; Grumbach, 1950; Lightbown, 1954). They also emphasize a difference, as yet undefined, between the mode of action of dihydrostreptomycin and that of the other antibiotics tested. This difference may explain the synergistic action of streptomycin when mixed with other antibiotics (see Demerec, 1948; Zinnemann, 1950).

The action of dihydrostreptomycin on Clostridium welchii

The resistance to dihydrostreptomycin of the two species of obligate anaerobes examined led to a fuller investigation of the factors underlying this resistance. The results are recorded in Tables 2 and 3. As observed with

Table 2. *Inhibition of growth of Clostridium welchii exposed to decreasing concentrations of dihydrostreptomycin*

Dihydro- streptomycin in ditch ($\mu\text{g./ml.}$)	Linear extent (mm.) of complete inhibition of growth, from culture diluted						
	1/1	1/10 ¹	1/10 ²	1/10 ³	1/10 ⁴	1/10 ⁵	1/10 ⁶
1000	0	1.5	2.0	2.5	3.0	3.0	6.0
500	0	2.0	2.0	2.5	3.0	3.0	4.0
200	0	0	1.0	1.0	1.0	1.0	2.5
100	0	0	0	0	0	0	1.0
50	0	0	0	0	0	0	1.0

Figures are average of three experiments.

numerous other organisms, the bactericidal concentration of dihydrostreptomycin for *Clostridium welchii* depends upon the inoculum size. The broth culture of *C. welchii* from which the various inocula were prepared contained approximately 30×10^8 viable organisms/ml. In the experiments with fluid media a Pasteur pipette delivering 29 drops/ml. was used, one drop constituting the inoculum; different dilutions were used to give inocula containing 1 to 1×10^8 organisms/drop. An inoculum of 1×10^6 organisms grew out in 1000 $\mu\text{g.}$ dihydrostreptomycin/ml. and an inoculum containing one organism grew out in 50 $\mu\text{g.}$ dihydrostreptomycin/ml. The results on solid media were similar.

Table 3. Relationship of inoculum size of *Clostridium welchii* to concentration of dihydrostreptomycin

Dihydro- streptomycin in medium ($\mu\text{g./ml.}$)	Growth of <i>C. welchii</i> when number of organisms in inoculum was				
	10^8	10^6	10^4	10^2	1
1000	+	+	—	—	—
500	+	+	—	—	—
200	+	+	—	—	—
100	+	+	+	+	—
50	+	+	+	+	+
25	+	+	+	+	+

+ = growth; — = no growth.

*The possible use of dihydrostreptomycin as a selective agent
for the isolation of Clostridium welchii*

An inoculum as small as one *Clostridium welchii* organism resists the action of dihydrostreptomycin at a concentration which is normally lethal to most facultative anaerobes growing anaerobically, e.g. an inoculum of 10,000 organisms of *Escherichia coli* is killed by dihydrostreptomycin 4–16 $\mu\text{g./ml.}$, depending on the strains used, when growing anaerobically. The results recorded in Table 4 reveal, however, that dihydrostreptomycin was less efficient as a selective agent than heating at 65° for $\frac{1}{2}$ hr. Furthermore, unless high concentrations of dihydrostreptomycin are used the growth of *Proteus*

Table 4. A comparison of the effectiveness of dihydrostreptomycin with that of heat treatment (65° for 30 min) for the isolation of *Clostridium welchii* from faeces

Faeces sample no.	Degree of growth of <i>C. welchii</i> on subculture from heated cooked meat medium	Growth of <i>C. welchii</i> on subculture from unheated cooked meat medium containing dihydrostreptomycin $\mu\text{g./ml.}$				
		1000	500	200	100	50
1 to 6	3	0	nt	nt	nt	nt
7	3	nt	1	2	2	nt
8	0	nt	0	0	0	nt
9	3	nt	Few cols.	1	1	nt
10	0	nt	0	0	0	nt
11	3	nt	0	0	0	nt
12	3	nt	1	2	2	nt
13	3	nt	nt	0	1	1
14	3	nt	nt	0	2	3
15	3	nt	nt	2	3	3
16	3	nt	nt	1	1	3
17	Few cols.	nt	nt	0	0	0
18	3	nt	nt	0	1	1

3 = heavy; 2 = moderate; 1 = light; 0 = no growth of *C. welchii*; nt = not tested. After a few hours on the bench *Proteus vulgaris* appeared on the fresh blood agar plate inoculated from the dihydrostreptomycin-cooked meat broths; the dihydrostreptomycin had exerted only a bacteriostatic effect on it.

vulgaris and *Streptococcus faecalis* is not inhibited. As these organisms occur frequently, together with *Clostridium welchii*, in infections, any advantage that might be gained from the use of dihydrostreptomycin is lost in the presence of *Proteus vulgaris* and/or *Streptococcus faecalis*.

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A Study of the Factors Influencing Non-genetic Variation in a Strain of *Fusarium oxysporum*

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SUMMARY: The colour variation of a strain of *Fusarium oxysporum* Schlecht ex Fr. was found to depend on the production of carotenoids and naphthoquinones. The carotenoids were only produced as a result of exposure to light, whereas the production of naphthoquinones, which were pH indicators, was mainly regulated by the carbon/nitrogen ratio of the medium. The morphogenetic effects of radiation and of carbon/nitrogen ratio were also considered. A low carbon/nitrogen ratio favoured chlamydospore formation and aerial mycelium, whereas ultraviolet radiation, and to a lesser extent light, promoted macrospore and sclerotium production.

The identification of *Fusarium* spp. has presented difficulties which are only now being slowly resolved. Appel, Wollenweber and their co-workers, who have made the greatest single contribution to *Fusarium* taxonomy, developed a system, in particular in *Die Fusarien* (Wollenweber & Reinking, 1935), which makes use of the presence or absence of microspores, chlamydospores and sclerotia, the shape and distribution of the macrospores and, to some extent, the colour and texture of the mycelium. They also introduced a cultural technique for producing the taxonomic features appropriate for identification. The small divisions which Wollenweber & Reinking erected as species have been challenged, in recent years, on two grounds. Brown (1928) and Snyder & Hansen (1940) showed that from a mass-isolate of a species it is possible to separate, by single conidial manipulation, isolates which by the criteria of Wollenweber would be distinct species, or would even fall into distinct sections of the genus. Buxton (1954) has taken this further by showing that within the section *Elegans* it is possible to make heterocaryons between morphologically distinct species, i.e. as recognized by Wollenweber, and has suggested that much of the extensive variation shown by *Fusarium oxysporum* may be due to a redistribution of the characters in the heterocaryon in the manner demonstrated by Pontecorvo (1952) for *Aspergillus nidulans* (Eidam) Wirt.

The second challenge to many of the Wollenweber species has come from the demonstration of the extreme sensitivity of the fungus to environmental factors, slight changes in environment having profound effects on the observed characters. As early as 1904 it was shown by Smith & Swingle that light affects the pigmentation of *Fusarium oxysporum*. Sideris (1925) examined the effect of pH value on pigmentation, and Brown and his co-workers in a series of papers (Brown, 1925, 1926, 1928; Brown & Horne, 1924, 1926) studied the factors affecting the production of various taxonomic characters in *F. fructigenum*. They developed a defined medium + agar simulating the potato agar then in use, and paid particular attention to the carbon/nitrogen ratio. Their

fungus produced only macrospores and was apparently not markedly affected by light. Ramsey & Bailey (1930) showed that exposure to ultraviolet radiation increased macrospore production, and Harter (1939) found that cultures of *Fusarium* spp. grown in the light showed increased macrospore length. Snyder & Hansen (1941) considered the effect of light on sporulation and on colour from the viewpoint of the taxonomist.

These observations have led *Fusarium* taxonomists to define more clearly the media and conditions under which the fusaria are grown, but difficulties still occur owing to the use of media such as potato glucose agar, which cannot by their nature be strictly defined, and by the difficulty of reproducing light of standard quality under varying laboratory conditions. With these considerations in view, an attempt has now been made to analyse the effects of various environmental factors, in particular light and nutrition, on a strain of *Fusarium oxysporum* under controlled conditions. The material used was an isolate derived from gladiolus corms, which proved to be particularly useful as it produced almost exclusively microspores or chlamydospores in the dark, and, under appropriate nutritional conditions, macrospores in the light. Associated with the morphological differences was a distinct colour difference.

The chemical nature of the pigments produced by fusaria is already understood in some instances. Ashley, Hobbs & Raistrick (1937) isolated two pigments from *Fusarium culmorum*, rubrofusarin, which is a methylxanthone, and aurofusarin, a highly insoluble substance, the constitution of which has not been determined. The pigments of the section *Martiella* were investigated by Arnstein & Cook (1947), Weiss & Nord (1949) and Ruelius & Gauhe (1950). The pigments successfully isolated and purified proved to be naphthoquinones. These authors, however, did not investigate the conditions which favour pigment production; this aspect has received special attention in the case of *F. oxysporum* in the following pages.

METHODS

All work was done with one single-spore isolate of *Fusarium oxysporum* Schlecht ex Fr. f. sp. gladioli (Massey) Snyder & Hansen, isolated from a corm of the gladiolus variety Wedgwood, being no. 87 characterized by Buxton (1955). Stock cultures were maintained in sterile soil, and on potato glucose agar. The inoculum for use in experiments was grown on a glucose + nitrate + Difco Bacto-Agar medium to diminish the carry-over of unknown substances from the potato glucose agar, and was kept in the dark at 25°. A microspore suspension of standard density was used for inoculation of tubes and plates. All glassware was acid-washed. The glass constant temperature tank used for these experiments was maintained at $25 \pm 0.5^\circ$ and was operated in a dark-room. The lid of the tank was of plywood with holes drilled to receive test-tubes, which dipped into the water.

The test-tubes used were all of the same make of Pyrex glass (1.5 cm. diameter, 15 cm. long) and contained 5 ml. of nutrient as an agar slope. The light source was a 400 W. MA/V mercury vapour lamp supplied by Philips

Electrical Ltd. and having the relative spectral energy distribution indicated in Table 1. The lamp was placed in a vertical position at one side of the tank at the focus of a paraboloid sheet-metal reflector. Tests with a photocell lowered into the tank and facing the lamp enabled the variations in light intensity, which were slight, to be followed, and tubes to be placed in such a manner as to receive equal illumination.

Table 1. *Relative spectral energy distribution of the MA/V mercury vapour lamp*

Wavelength (Å) ...	3665	4047	4078	4356	4916	5461	5780
Watts	5.8	4.8	1	9.9	0.3	15.3	17
Colour	Ultra-violet	Violet			Blue-green	Yellow	

Tubes, wrapped in opaque black paper were placed at the back of the tank. Unwrapped tubes were placed in front in such a manner as not to interfere with each other and to receive equal illumination. Three replicates were provided for each treatment. Departures from the experimental procedure described are indicated in the text.

RESULTS

General behaviour of cultures in light and darkness

In preliminary work the fungus was grown on a wide variety of agar media under light and dark conditions. The development of the cultures is recorded in Table 2. It was concluded that light promoted the formation of orange pigments and of macrospores, whereas darkness favoured the formation of diffusible red and purple pigments and chlamydo-spores at a later stage in the history of the colony, provided nutritional conditions were suitable.

Table 2. *Observations on the development of the fungus using a wide variety of media*

Days	Light	Darkness
4	Orange non-diffusible pigment Macrospores abundant No sclerotia No chlamydo-spores	No pigment Macrospores rare or absent No sclerotia No chlamydo-spores
20	Orange non-diffusible pigment Macrospores rare except in the form of sporodochia on some media (e.g. Yeastrel agar) Sclerotia on some media (e.g. Czapek-Dox + 1.5 % glucose + agar) No chlamydo-spores	Red or purple diffusible pigments on some media (e.g. potato glucose agar) Macrospores rare or absent No sclerotia Chlamydo-spores abundant on some media (e.g. yeast + glycerol + agar)

In the next experiment the effect of the nitrogen source upon pigmentation and morphological response was investigated. The basal medium consisted of the following constituents (% w/v): Difco Bacto-Agar, 1.5; D-glucose, 2;

MgSO₄·7H₂O, 0.05; KCl, 0.05; Na₂HPO₄·12H₂O, 0.1; trace elements as recommended by Beadle & Tatum (1945); glass-distilled water. The nitrogen content of the medium was varied so as to provide the following treatments (% w/v): NaNO₃, 0.4 (0.065 N); NaNO₃, 0.04 (0.007 N); asparagin, 0.4 (0.075 N); asparagin, 0.04 (0.008 N); Bactotryptone, 0.4 (0.053 N). Potato glucose agar was included in this treatment as a control because it is extensively used for the growth and characterization of *Fusarium* spp. The effect obtained with NaNO₃ as the nitrogen source is indicated in Table 3. The cultures on the medium containing 0.04% asparagin, and on potato glucose agar, behaved similarly to those with 0.04% NaNO₃; whereas those with 0.4% asparagin and 0.4% Bactotryptone behaved similarly to those with 0.4% NaNO₃. It was concluded that the carbon/nitrogen ratio was of greater importance than the nature of the nitrogen source in promoting the observed effects.

Table 3. *Appearance of colonies grown on basal medium with sodium nitrate as the nitrogen source*

NaNO ₃ (% w/v)	Light	Darkness
0.04	Orange	Purple
	Flat	Flat
	Macrospores common	Macrospores absent
	No sclerotia	No sclerotia
	No chlamydo spores	No chlamydo spores
0.4	Orange	White
	Aerial	Aerial
	Macrospores common	Macrospores absent
	Sclerotia in old cultures	No sclerotia
	No chlamydo spores	Chlamydo spores in old cultures

To test this an experiment was carried out keeping the nitrogen concentration constant and varying the glucose concentration. The basal medium was as used in the previous experiment with the addition of 0.1% (w/v) NaNO₃. The glucose concentrations used were 0.25, 1, 2 and 5%. Also employed were media with 0.25 and 5% glucose with the addition of the vitamin mixture recommended by Pontecorvo (1952). This was to test the possibility that some of the differences observed in cultures on defined media such as Czapek-Dox and on media such as potato glucose agar might be due to the presence of vitamins in the latter. The results are indicated in Table 4. The effects of the addition of vitamins proved slight.

Pigments produced only in the light

Having determined the conditions under which pigments were produced on defined media + agar, it was necessary to learn something of their chemical nature before considering their possible significance as regards the general behaviour of the fungus. The orange pigment produced in the light by young actively growing colonies did not diffuse into the medium, did not appear to be affected by changes in the composition of the medium, and was not readily extracted by any of the commonly used solvents available. The possibility of

the pigment being a carotenoid or mixture of carotenoids was therefore considered.

Cultures were grown on potato glucose agar in Petri dishes and after 14 days growth, during which they were exposed to daylight supplemented by continuous illumination from a mercury vapour fluorescent tube at 15 cm. distance, the mycelium was removed from the surface of the agar. After blotting with

Table 4. *Effect of varying glucose concentration*

Glucose (%, w/v)	Light	Darkness
0.25	Pale orange Macrospores abundant Chlamydospores in old cultures No visible oil droplets Sclerotia absent	White Macrospores absent Chlamydospores in old cultures No visible oil droplets Sclerotia absent
1.0	Orange Macrospores common No chlamydospores No visible oil droplets Sclerotia common	White, with a little blue Macrospores absent Chlamydospores in old cultures No visible oil droplets Sclerotia absent
2.0	Strong orange Macrospores common No chlamydospores Oil droplets present Sclerotia common	Purple Macrospores absent No chlamydospores Oil droplets present Sclerotia absent
5.0	Strong orange Macrospores common No chlamydospores Oil droplets present Sclerotia absent	Intense purple Macrospores absent No chlamydospores Oil droplets present Sclerotia absent

filter-paper the mycelium was ground with anhydrous sodium sulphate. The mixture was then repeatedly extracted with peroxide-free diethyl ether until further extracts were colourless. The extracts were combined, dried with anhydrous sodium sulphate, evaporated under reduced pressure, taken up in light petroleum and chromatographed on a column of methanol-inactivated alumina.

Several fractions were found to be present and spectrophotometric readings were taken for two of the major fractions. The fractions proved to be non-saponifiable with 60% KOH (w/v), and were epiphasic on addition of light petroleum. It was concluded from the solvent properties, spectrophotometric data and behaviour on saponification that these fractions were carotenoids, and it seems probable that the entire orange colour of the mycelium in the light is due to a mixture of carotenoids.

The cultures which received glucose in high concentrations were the most brightly coloured, in agreement with the findings of Goodwin (1952). To find how long the cultures needed to be irradiated to produce pigmentation, 3-day cultures on Czapek-Dox agar (glucose 2%) in Petri dishes were exposed to the mercury vapour lamp at 60 cm., the temperature being kept at 20–21° with an electric fan. Exposure for 1 hr. produced perceptible pigmentation and

exposure for 10 hr. a strong orange colour. The greater part of the colour production took place in darkness during the 24 hr. following irradiation. Colour production was limited to the mycelium that was irradiated, later-formed mycelium being colourless. Old mycelium largely loses the power of producing carotenoids, since exposure for 14 hr. of 10-day cultures failed to cause pigmentation.

These findings are in agreement with the recent investigations of Zalokar (1954, 1955) on the photoactivation of carotenoid synthesis in a strain of *Neurospora crassa*. A photoactivation of carotenoid formation in *Pyronema confluens* has also been found (Carlile & Friend, to be published), and it seems probable that this is the explanation for the orange colorations reported for many fungi when grown in the light.

Pigments produced mainly in darkness

Cultures grown in darkness on some media produced a red-purple pigmentation. The pigment developed late in the history of the colony, after mycelial growth had largely ceased. Pigment is present in the mycelium and diffuses into the medium, whether agar or liquid. In media with a high carbon/nitrogen ratio these pigments are also formed to some extent in the light, but considerably later than the carotenoids.

Extracts of pigment were obtained by disintegrating the cultures grown on potato agar in a Waring blender with chloroform. Water and a little acetic acid were next added to the blend, which was then centrifuged. A deep red solution of pigment in chloroform was thus obtained. An alternative method of extraction was to leave chloroform on the cultures overnight. The crude pigment was very soluble in chloroform, ether, acetone, and ethanol, and fairly soluble in water. It passed from chloroform into aqueous solutions of ammonia, sodium hydroxide, or sodium carbonate, but not into sodium bicarbonate, acetic acid or hydrochloric acid. The crude pigment was red in acid solution, and purple in alkaline solution, the end-point of the colour change being about pH 8. In aqueous solutions the pigment decolorized bromine water, and with lead acetate in methanol, an intense violet coloration was obtained. Paper chromatography indicated that the pigment was a mixture. The reactions of crude pigment suggest a mixture of substituted 5 : 8-dihydroxy-1 : 4-naphthoquinones (Kuhn & Wallenfels, 1939). Complex mixtures of such substances have been found in the closely related fusaria of the section *Martiella* by other workers (Arnstein & Cook, 1947; Weiss & Nord, 1949; Ruelius & Gauhe, 1950). Through the courtesy of Dr E. C. Bate-Smith and Professor F. F. Nord it was possible to compare the crude pigment with samples of fusarubin, javanicin, and solanione. When the pigment was chromatographed with these substances as markers it was found that some of the fractions were closely similar in appearance and R_f values to the *Martiella* naphthoquinones, suggesting a close similarity if not actual identity.

It was clear from earlier experiments that media containing a considerable excess of carbon over nitrogen promoted the development of purple pigment. It was not clear, however, at what level of carbon/nitrogen ratio this effect

becomes observable. Moreover, on some media the colour developed as a blue-green ring, reminiscent of an effect frequently observed in *Fusarium caeruleum*. To investigate these phenomena, Czapek-Dox medium + agar, but without sucrose, was used as a base, and glucose was added over the following ten concentrations (% w/v): 1, 1.5, 1.8, 1.9, 2.0, 2.1, 2.2, 2.5, 3 and 4. Twenty ml. portions of each medium were added to three Petri dishes (8.5 cm. diameter), which were centrally inoculated with a spore suspension and incubated at 25°. The time of appearance of the pigment and its distance from the centre of the dish were noted (Table 5). There was a clear relationship between the glucose

Table 5. *The effect of glucose concentration on the diameter of the non-pigmented area*

Glucose (%, w/v)	Average diameter of non-pigmented area (cm.)
1.0	No pigment
1.5	6.1
1.8	5.5
1.9	5.3
2.0	5.1
2.1	4.9
2.2	4.6
2.5	4.0
3.0	3.0
4.0*	0.5

* Reading taken at 5 days; subsequent inward diffusion of pigment obliterated colourless areas. All other readings at 10 days.

content and the diameter of the non-pigmented area. With increasing glucose concentration the colour of the pigment changed from blue to red. The addition of acid or alkali to the plates showed that this was a pH effect, high nitrate making the medium alkaline and high glucose, acid. After the ring had appeared, inward diffusion decreased the diameter of the colourless area and sometimes obliterated it. The effects of glucose and nitrate concentrations are further indicated in Table 7.

The development of pigment as a coloured ring, the diameter of which is related to the carbon/nitrogen ratio of the medium is of interest in illustrating the extent to which diffusion of nutrients probably takes place in the culture plate system. It is suggested that with an excess of glucose the rapid exhaustion of nitrate leads to an inward diffusion of nitrate, thus altering the carbon/nitrogen ratio near the periphery to a value where pigment formation will occur. Observations were carried out in which the nutrient distribution in 8-day cultures on media with different glucose concentrations (Table 6) was examined by means of the diphenylamine test for nitrate and the Fehling's test for glucose; the initial nitrate concentration was 0.2 % (w/v). The data obtained support the diffusion hypothesis, and suggest that a changing ratio between nutrients is a possible explanation of various zoning phenomena in Petri dishes.

The production of naphthoquinones can be regarded as a metabolic shunt operating at high glucose concentrations. Such a mechanism has been proposed as the general mode of origin of the 'Raistrick substances' formed by many fungi. On the other hand, in view of the known biological activity of many naphthoquinones it should not be assumed that they are wholly functionless.

Table 6. *Glucose and nitrate distribution in 8-day cultures on media differing only in glucose concentration*

Glucose concentration (%, w/v)	Distance from centre of plate in cm.							
	Fehling's test				Diphenylamine test			
	1	2	3	4	1	2	3	4
1	Blue	Blue	Blue	Blue	+	+	+	+
1.5	Blue	Blue	Blue	Green	—	—	—	—
2	Blue	Blue	Blue	Green	—	—	—	—
3	Blue	Blue	Green	Red	—	—	—	—
4	Green	Yellow	Yellow	Red	—	—	—	—

Morphological behaviour in light

Cultures of the strain used invariably produced microspores. When exposed to summer daylight or to radiation from the mercury vapour lamp an abundance of macrospores occurred in association with the carotenoid pigmentation. Examination of the mode of origin of macrospores and microspores showed that they were essentially similar, the difference being that in macrospore formation a great elongation of the spore rudiment occurred, after which septa developed. The distinction between macrospores and microspores was not absolute, a continuous gradation of spore length and septation being present in any one culture. In view of this it was decided to regard spores longer than $21\ \mu$. as macrospores. Macrospores were produced in the middle of a young colony as a pionnotal mass. As colonies grew an increase in aerial mycelium and a decrease in macrospore production occurred. In consequence, the contrast between cultures grown in light and darkness was most marked in young cultures, and for this reason it was not easy to obtain absolute figures for macrospore production/colony.

In older cultures grown on media with a low carbon/nitrogen ratio which were exposed to light for 3–4 weeks, there occurred what might be called secondary macrospore production. Discrete areas of fresh macrospore production were found scattered over the surface of the thallus. In these cases every gradation existed between a liquid drop consisting of a dense suspension of macrospores, a soft 'pustule' containing both mycelium and macrospores (a sporodochium), and a hard tangle of mycelium (a sclerotium); the precise conditions which lead to the predominance of one or the other are not clear. It is of interest that the orange pigmentation disappeared from the mycelium around a 'pustule' and became concentrated in the 'pustule'. On inspection the cytoplasm was found to have disappeared from the decolorized hyphae; a cytoplasmic flow into the developing 'pustule' is therefore suggested.

Morphological behaviour in darkness

In cultures grown in darkness the almost total absence of macrospores was striking. When present at all they rarely exceeded 1 % of the total spore production, even at the centre of the colony. On a few media, such as malt extract agar, they sometimes reached 5 %; the reason for such exceptions is unknown. Cultures grown in the dark have not been known to produce either sporodochia or sclerotia. Cultures on a medium with low glucose/nitrate ratio produced abundant chlamydospores at 25°, and in their formation the surrounding mycelium became emptied of its cytoplasmic contents. At high glucose/nitrate ratios chlamydospores were not found but there was an accumulation of oil droplets in the mycelium of the older parts of the colony (Table 7).

Table 7. *The effect of glucose and nitrate concentration on the production of pigment, oil droplets and chlamydospores in cultures grown in darkness*

Glucose/NaNO ₃	Glucose (%, w/v)	NaNO ₃ (%, w/v)	Pigment	Oil	Chlamydo- spores
2	0.2	0.1	—	—	+
2	1.0	0.5	—	—	+
5	1.0	0.2	—	—	+
5	2.0	0.4	—	—	+
7.5	1.5	0.2	Ring	—	+
10	1.0	0.1	Ring	—	+
10	2.0	0.2	Ring	—	+
12.5	2.5	0.2	Ring	—	+
15	3.0	0.2	Ring	+	Few
20	2.0	0.1	+	+	—
20	4.0	0.2	+	+	—
50	1.0	0.02	+	+	—
50	5.0	0.1	+	+	—

Pigment, spore production and ultraviolet irradiation

All young cultures exposed to light produced carotenoids and macrospores, whereas all cultures grown in the dark produced neither. An attempt was therefore made to ascertain whether a causal relationship existed between carotenoid formation and macrospore production. An 80 W. Mazda lamp emitting 97 % of its radiation in the ultraviolet region and 75 % at 3650 Å. was highly effective in promoting macrospore production. Exposure for 1 hr. of a 3-day culture at a distance of 30 cm. (temperature 22–25°) was sufficient to bring about a massive production of macrospores, about 48 hr. being required for complete development. This exposure did not cause carotenoid formation. The macrospore formation occurred only within the limits of growth at the time of irradiation. Exposure for 10 hr. of similar cultures to the mercury vapour lamp which emitted mainly visible light, caused intense pigment formation but no macrospore production. It is concluded that the photo-activation of carotenoid synthesis and the induction of macrospore formation are unrelated phenomena requiring different wavelengths.

Exposure of a 10-day culture for 1 hr. to a mercury vapour lamp emitting 87 % of its radiation at 2536 Å. brought about the formation of white 'pustules' consisting of macrospores after a further 9 days of incubation. The importance of ultraviolet radiation in promoting sporulation in fungi has been recognized since the work of Stevens (1928), and was established for fusaria by Ramsey & Bailey (1930). However, in papers by Harter (1939) and Snyder & Hansen (1941) the promotion of macrospore formation is regarded as being due to visible radiation. The notorious variability within *Fusarium* spp. and the differences between species makes it difficult to compare the work of various authors. However, from the work reported in the present paper, it appears that it is the ultraviolet component of radiation from either the mercury vapour lamp or from daylight that is mainly responsible for the promotion of macrospore production in *F. oxysporum* after short exposures. It is possible that long exposures to visible radiation may have similar but weaker effects as compared with ultraviolet radiation. A further study of the effectiveness of different wavelengths on promoting sporulation is clearly desirable.

DISCUSSION

The pigments produced by the strain studied were shown to fall into two distinct groups, one group being the result of photoactivation and the other being due to high glucose concentrations in the medium. Similar work is being carried out with other species of fusaria, and it is hoped that such an approach will enable colour to be more usefully employed as a taxonomic criterion in this genus.

The demonstration that, although production of both carotenoids and macrospores is brought about by daylight, either effect can be obtained in the absence of the other, indicates that in this species there is not an obligate relationship between the two effects. Evidence has been obtained that a comparable situation exists as regards carotenoid formation and reproduction in *Pyronema confluens* (Carlile & Friend, to be published). Such studies are of significance in view of suggestions that carotenoids play an important part in reproduction.

In recent years the problem of carotenoid biosynthesis has been attracting considerable attention, and attempts to identify possible precursors have so far proved unsuccessful. It would appear that organisms such as *Fusarium oxysporum* and *Neurospora crassa* in which carotenoid synthesis can be triggered by brief exposures to light could be usefully employed in investigating this problem.

Finally, the blue ring phenomenon described gives an elegant demonstration of the probable significance of radial diffusion of metabolites in the Petri dish system, a topic that has so far received little attention.

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The Influence of certain Micro-organisms on the Formation of Perithecia by *Chaetomium globosum*

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SUMMARY: The localized formation of perithecia in colonies of *Chaetomium globosum* growing in association with certain other micro-organisms (e.g. *Aspergillus fumigatus*) is associated with the presence of organic phosphates, especially phosphoglyceric acid, in the medium. These phosphates are normal metabolic products of the associated micro-organism, and only when they diffuse into the medium and locally reach a critical concentration does the fruiting response follow.

The stimulatory effect of one fungus on the sporulation of another was reported by Heald & Pool (1908), and has since been noted frequently. The subject was reviewed by Asthana & Hawker (1936) who carried out one of the most detailed studies of the subject. Working mainly with *Melanospora destruens* Shear they found that the intensity of sporulation of this fungus in mixed cultures was governed by a number of interdependent factors, one of which was the rate of production of a chemical stimulant of sporulation by the associated organism. Buston & King (1951), who had observed stimulation of production of perithecia of *Chaetomium globosum* Kunze following contamination by a strain of *Aspergillus fumigatus* Fresen, obtained active extracts from the mycelium of the latter organism and from the staled medium, but were unable to isolate or characterize the substance responsible. Hawker (1948) demonstrated the stimulant effect of glucose-1-phosphate and of fructose-1, 6-diphosphate on the fruiting of *Melanospora destruens*, but Buston & King (1951) showed that these compounds, although possibly present, did not entirely account for the activity of the *Aspergillus* extract. Buston, Jabbar & Etheridge (1953) applied a chromatographic method to the investigation of a 'fruiting factor' present in an aqueous extract of jute (Buston & Basu, 1948), and concluded that its activity could be accounted for by the presence of glucose-6-phosphate, fructose-1, 6-diphosphate and calcium ions in a particularly favourable balance of concentrations. The same chromatographic technique has now been applied to extracts of *A. fumigatus* and other micro-organisms which stimulate fruiting of *Chaetomium globosum* in mixed culture.

RESULTS

The strain of *Chaetomium globosum* was that previously used in this laboratory (Basu no. 79); the strain of *Aspergillus fumigatus* was isolated by Buston & King from a chance infection. The cultural methods used were those previously described (Buston & King, 1951). Extracts of the mycelium of *A. fumigatus*, grown in liquid media, were made with cold 0.1 N-NaOH in the presence of

a little ether, and after neutralization with HCl were evaporated almost to dryness *in vacuo* at as low a temperature as possible. The residue was taken up in 95 % (w/w) ethanol in water, undissolved NaCl removed, and the solution again evaporated *in vacuo*, after which the residue was dissolved in a little water. From the staled liquid media, material was obtained in a similar manner, after removal of the greater part of the inorganic salts by precipitation with ethanol. The final products still contained small amounts of inorganic salts.

Preliminary tests having shown that these extracts accelerated the formation of perithecia of *Chaetomium globosum*, suitable portions were examined for the presence of organic phosphates by paper chromatography as described by Buston *et al.* (1953). The mycelial extract was found to contain glucose-1-phosphate, fructose-1, 6-diphosphate and larger amounts of phosphoglyceric acid; the staled medium contained phosphoglyceric acid and a trace of fructose-1, 6-diphosphate. Phosphoglyceric acid has not been studied hitherto in connexion with perithecial formation, and experiments showed that like the hexose phosphates it has a stimulant effect within certain limits of concentration (Table 1). It seemed reasonable, therefore, to conclude that the observed

Table 1. *Effect of phosphoglyceric acid (Ca salt) on sporulation of Chaetomium globosum*

Phosphoglyceric acid (Ca salt) added (mg./100 ml. medium)	Perithecial frequency* (5th day)
20	0.6
30	1.2
40	2.2
50	2.8
60	1.4

* Asthana & Hawker (1936). Average number of perithecia/field; counts made on three fields in each of ten sectors.

stimulation of fruiting was due to the presence of suitable amounts of these organic phosphates, and especially of phosphoglyceric acid, in the medium. However, it must be borne in mind that in previous experiments, although known amounts of the various organic phosphates were added to the original medium, nothing is known of any change in concentration that may occur subsequently. Thus neither the *effective* concentration at the moment when fruiting is initiated, nor the exact stage of development of *C. globosum* at which the chemical stimulus can evoke the fruiting response, is known. For this reason estimations of the amount of organic phosphate in the medium at the moment when perithecia are first visible may be of little significance; nevertheless, it seemed of interest to attempt such estimations, and for this purpose portions of the agar medium were removed from positions adjacent to the fruiting zones as soon as perithecia were seen, and the phosphates were separated on a chromatogram. Total organic phosphate was estimated in

eluates from the chromatogram according to the method of Berenblum & Chain (1938). In two such experiments the local concentration of organic phosphate was estimated as equivalent to *c.* 30 mg. phosphoglyceric acid/100 ml. of medium.

The stimulation of fruiting of *Chaetomium globosum* in the presence of other micro-organisms was studied by Basu (1947), who tested the influence of fifteen different species grown side by side with *C. globosum* on agar plates. Under the conditions which he used, Basu found no effect on the sporulation of *C. globosum*; perithecia appeared at the same time as in the controls, and developed from the centre of the colony rather than at the edges nearest the second organism. Among the species tested by Basu was a strain of *Aspergillus fumigatus*, and in view of the results now obtained with this organism some further tests were made both with our strain and with that used by Basu. It was found that the discrepancies between our results and those of Basu were due in part to the different distances between the colonies of the two organisms. Maximum fruiting resulted when the centres of the colonies were about 20 mm. apart; at 50 mm. the effect was barely perceptible (Table 2). Dr Basu has kindly repeated his experiments with *A. fumigatus*, and he now reports that 'the response was more marked at 20 mm. than at 40 mm'.

Table 2. *The effect of distance between colonies of Chaetomium globosum and Aspergillus fumigatus on the formation of perithecia by C. globosum*

C. globosum was inoculated 1 day before *A. fumigatus*

Distance between centres of colonies (mm.)	Perithecial frequency (8th day)	
	In 90° sector nearest colony of <i>A. fumigatus</i>	Average over whole colony
20	12	3.9
30	10	2.9
40	9 (small)	2.3
50	3 (small)	2.3

The fruiting of *Chaetomium globosum* is also affected by the relative age of the two colonies, and the greatest response is found when this organism is inoculated one day earlier than the *Aspergillus fumigatus*, at a distance of 20 mm. When the *Chaetomium globosum* is inoculated 4 days earlier, growth of *Aspergillus fumigatus* is completely inhibited.

The influence of some of the other organisms tested by Basu was also re-examined; in two instances a definite positive response was obtained when the distance between the colonies was suitable. In one other case a feeble response was observed, but the rest of the organisms gave negative results at all the distances tested. The distribution of organic phosphates in the mycelia of these organisms, and in their staled media, was also examined, and the results are summarized in Table 3. In all instances mycelial extracts contained organic phosphates, phosphoglyceric acid predominating, but organic phosphate was detected in the media only in those instances where a positive fruiting response had been observed.

Table 3. *Phosphorylated compounds in mycelia of certain micro-organisms, and in staled media*

Organism	Strain	Phosphates in		Relative degree of fruiting response by <i>C. globosum</i>
		Mycelial extract	Staled medium	
<i>Aspergillus fumigatus</i>	B. & K.	FP PGA G-1-P	FP (f.) PGA	+++
<i>A. fumigatus</i>	Basu	n.i.	PGA	++
<i>A. flavus</i>	Kew	FP PGA XP	FP PGA XP	++
<i>A. terreus</i>	Kew	FP PGA	FP PGA	++
<i>A. ustus</i>	I.C.	PGA (f.) XP	n.d.	—
<i>Penicillium citrinum</i>	Kew	PGA (f.) XP	n.d.	—
<i>P. islandicum</i>	I.C.	FP PGA (tr.)	n.d.	—
<i>P. notatum</i>	Kew	FP PGA G-6-P	FP PGA	+
<i>Trichoderma viride</i>	I.C.	PGA (tr.)	n.d.	—

Sources of strains: B. & K. = Buston & King, 1951; Basu = Basu, 1947; Kew = Commonwealth Mycological Institute; I.C. = Department of Mycology, Imperial College.

Phosphates: FP = fructose-1, 6-diphosphate; PGA = phosphoglyceric acid; G-1-P = glucose-1-phosphate; G-6-P = glucose-6-phosphate; XP = unidentified organic phosphate; (f.) = faint; (tr.) = trace. n.d. = no phosphate detected; n.i. = not investigated.

Relative degree of fruiting response is scored + + +, + +, + in decreasing order of magnitude; — = no response.

DISCUSSION

The effect of phosphoglyceric acid in stimulating production of perithecia has not been recorded hitherto, but in view of the close relationship between this substance and the hexose phosphates it may be presumed that the mechanism of its action is the same. The occurrence of glucose-1-phosphate in the mycelium of *Aspergillus fumigatus* was somewhat unexpected. Buston & King (1951) noted the relative resistance to mild acid hydrolysis of extracts of *A. fumigatus* as compared with active jute extracts; this is in agreement with the comparative stability of phosphoglyceric acid, which does not occur in jute extract.

Although all the mycelia examined were found to contain organic phosphates, there were considerable differences in the amounts present; this probably merely reflects a difference in the rate and not the pathway of metabolism. What is more significant in regard to the present problem is the variation in the amount of phosphate which passes into the surrounding medium. This might be due to differences in the permeability of the mycelial membrane as well as to differences in the concentration of phosphate within the tissues, and

since production of perithecia seems to require a rather narrow range of concentrations of the various organic phosphates (alone or in combination) it is reasonable to conclude that the rate of diffusion of these substances from the mycelium and through the medium will play a major part in deciding whether any stimulation of fruiting shall occur. The observed differences in response with different inter-colony distances can also be explained as a diffusion-concentration phenomenon. In all cases studied in which the presence of a second organism caused accelerated or increased production of perithecia by *Chaetomium globosum*, the effect was associated with the presence in the medium of phosphoglyceric acid and other organic phosphates.

We are greatly indebted to Dr S. N. Basu for helpful collaboration and discussion.

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ADDENDUM

Our colleague Mr M. W. McDonough has shown that *Aspergillus fumigatus* excretes organic phosphates into the medium in the earliest stages of its growth, and the fruiting response of a neighbouring colony of *Chaetomium globosum* may follow later even if the colony of *Aspergillus fumigatus* has been removed in the meantime. In his experiments that portion of the agar containing the latter colony was cut out on the second or third day after inoculation, and the gap in the medium immediately refilled with fresh agar (in order to avoid stimulation of fruiting of *Chaetomium globosum* being caused by

the presence of the cut edge of the agar). When the two colonies were inoculated on the same day and *Aspergillus fumigatus* removed 2 days later, the perithecial frequency (counted on the 8th day) in the quadrant of the *Chaetomium globosum* colony nearest the position formerly occupied by *Aspergillus fumigatus* was 10·2, in the other three quadrants, 8·7. When *A. fumigatus* was removed on the third day, the corresponding numbers were 14·7 and 8·9; increased formation of (immature) perithecia could be seen on the fourth day.

When the colony of *Aspergillus fumigatus* was excised on the second day, about two-thirds of the total organic phosphorus was found to be within 8 mm. of the position of the colony, and 2 days later it had diffused to give a fairly equal concentration through 25 mm. When the colony was allowed to grow for another day, approximately four times as much organic phosphate had been excreted.

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Automatic Control of pH Value in Cultures of Micro-organisms

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SUMMARY: Equipment for the automatic control of pH value in cultures of micro-organisms is described. The apparatus was designed for a 2 l. scale continuous culture apparatus. The equipment will continuously control pH value with an accuracy of ± 0.05 unit for periods of many hundred hours. The pH value may be changed at will by turning a knob on the controller. It is sufficient to check the pH meter standardization once every day or two. The main components of the apparatus are standard commercial products.

The control of pH value is a factor of fundamental importance in the cultivation of micro-organisms and must, in general, be controlled within narrow limits to achieve the optimum conditions. To enable one to control the pH accurately over a wide range and to change the pH at will it is essential to use automatic control instruments. Apparatus for the automatic control of pH value is of recent development and requires instruments of relatively complicated design. Morton (1932) seems to have been about the earliest to describe an automatic pH control apparatus. A pH-controlling apparatus applicable to pure cultures of micro-organisms was described by Longworth & MacInnes in 1935. The complicated and delicate nature of their apparatus, which had to be built in the laboratory, must have precluded it from coming into general use. The commercial development of electronic instruments has made automatic pH control instruments much more readily available. Recently, several groups of workers have reported the use of instruments for the automatic control of pH value (Neish & Ledingham, 1949; Kempe, Halvorson & Piret, 1950; Hosler & Johnson, 1953; Wheat, 1953; Lakata, 1954). The present paper describes equipment suitable for controlling accurately the pH value of cultures of micro-organisms for periods of many hundred hours with complete freedom from contamination. Included are details of component design and operating technique not previously described.

APPARATUS

The pH-control equipment was used on a reactor for the continuous culture of micro-organisms. A diagram of the whole assembly is given in Fig. 1. The points of the reactor design relevant to this work are as follows. The vessel was a Pyrex glass pipe with stainless steel base and head plates. The volume of culture was about 2 l. It had automatic temperature control. The agitation was by means of a vaned disk impeller rotated at 500-1000 r.p.m. The impeller, of which the diameter was approximately half that of the vessel, had eight vanes, 15 mm. in height.

The equipment used for pH control in the reactor consists of a pH meter (model 23A, Electronic Instruments Limited, Richmond, Surrey), the output of which besides operating the usual galvanometer is fed to a recorder/con-

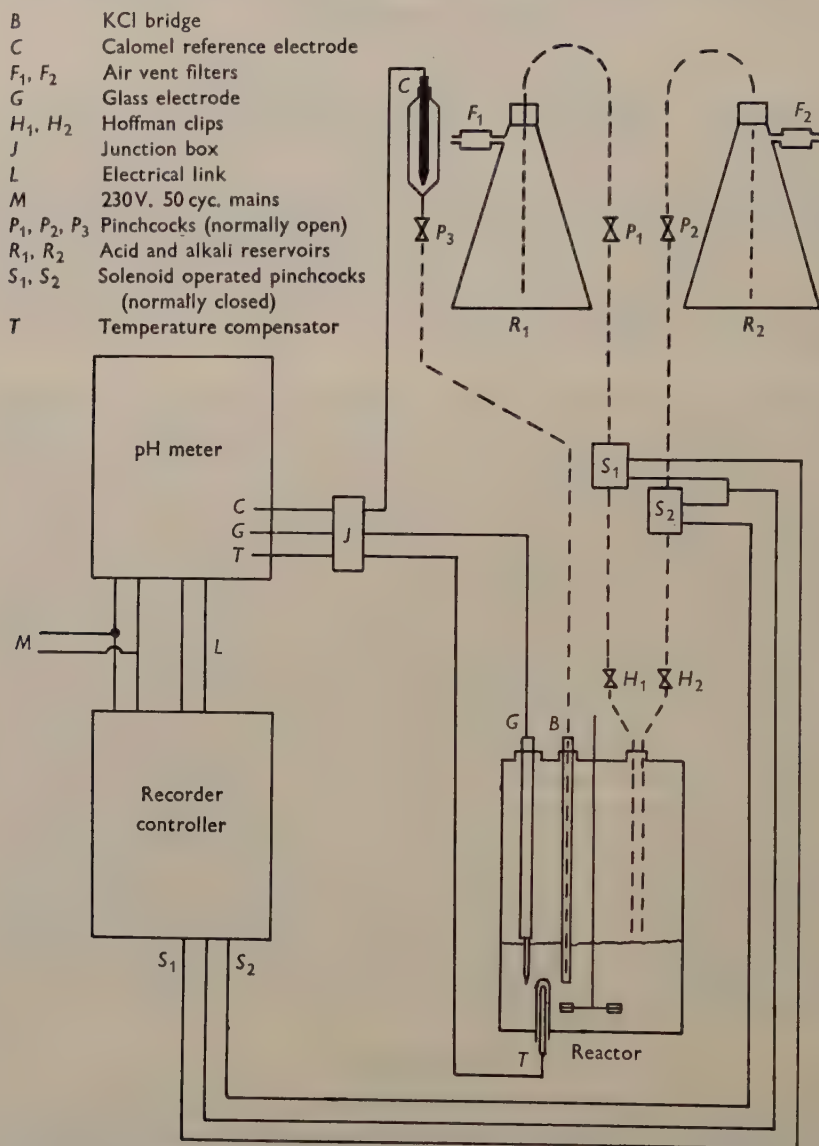


Fig. 1. Diagram of apparatus for automatic pH control of reactor.

troller (type 153, Elektronik electric contact controller, Honeywell Brown Ltd., Greenford, Middlesex). The latter instrument is a self-balancing potentiometer which gives a continuous record of the pH value as given by the meter, and in addition operates two mercury switches connected to solenoid valves or other

control gear for admitting acid or alkali to the reactor. This enables the pH value of the culture fluid in the reactor to be kept constant, within limits, at any setting of the controller, the setting being immediately adjustable over the range 0–10 pH units by the turn of a knob. The two instruments are housed in a light metal frame separate from the reactor frame to ensure freedom from vibration. As an alternative to the pH measuring and controlling instruments which we have used, we considered an automatic titrimeter, one of the types which have recently been produced commercially for acid-base titration. A preliminary trial of such an instrument indicated that it had neither the accuracy nor the long-term stability which we required for our purpose.

Electrodes. The pH electrodes (supplied by Electronic Instruments Ltd.) consist of a spear type glass electrode with Ag-AgCl half cell and a calomel reference electrode, only the former being within the reactor. The reference electrode is outside and above the reactor, contact with the culture fluid being made with a long KCl bridge. The bridge consists of glass and silicone tubing with an internal diameter of 3 mm. It is filled with saturated KCl solution and terminates within the reactor in a porous ceramic plug which is a bacterial filter and at the other end in a 100 ml. separatory funnel used as KCl reservoir and carrying the reference electrode. The reservoir is about 120 cm. above the reactor in order to provide the necessary head to cause a slight flow (about 5 ml./day) of KCl through the ceramic plug. The glass electrode is fitted in a metal tube in such a way that most of the glass part is outside the tube. The ebonite cap and the cable are inside the metal tube and isolated from the reactor contents by a rubber bush which makes a liquid-tight seal. The spear-type electrode was selected instead of the bulb type because the former can be inserted through a rubber bush without risk of damage. The glass electrode in its holder and the KCl bridge are fitted in the head plate of the reactor by means of rubber bungs. The bungs are held in ports consisting of short lengths of externally threaded stainless steel tubing. The bungs are kept tightly in place by threaded brass caps fitting on the stainless steel tubing. Alternatively, the KCl bridge may be fitted in the base of the reactor. We tried this arrangement but prefer the other as, on the head plate in our installation, the glass part of the KCl bridge is less liable to accidental breakage. The electrodes are arranged so that their tips are at about the 1300 ml. level, that is, well below the normal working level of *c.* 2 l. The standard electrodes as received from the manufacturers had 90 cm. leads, too short for use on the reactor. They are coupled to the pH meter by means of extension leads connected with a normal junction block. A metal-sheathed cable is used for the glass electrode. No trouble has been experienced from this simple method of connexion.

In addition to the electrodes, a resistance thermometer is also connected to the pH meter through the junction block. This resistance thermometer, which is part of the normal temperature compensating device of the pH meter, is housed in a pocket in the base of the reactor. The reference electrode remains at room temperature. The difference in temperature between the two electrodes is compensated for by the isopotential control of the pH meter.

Arrangement for admission of acid and alkali to the reactor

Acid and alkali reservoirs are installed some 90 cm. above the reactor and feed by gravity through glass and silicone tubing. Gravity feed was preferred to pressure feed as it allows a safer installation. The reservoirs consist of Buchner flasks with a useful capacity of 6 l. The side arms carry small air filters packed with fine Fibreglass. The reservoirs are calibrated so that the

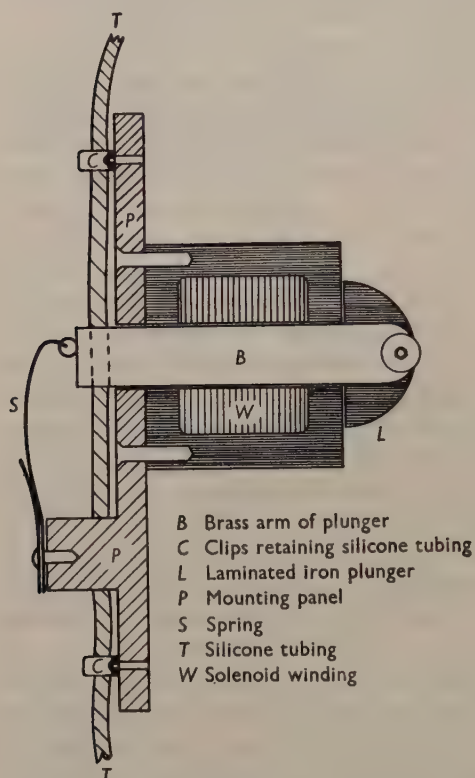


Fig. 2. Solenoid-operated pinchcock shown in the open position.

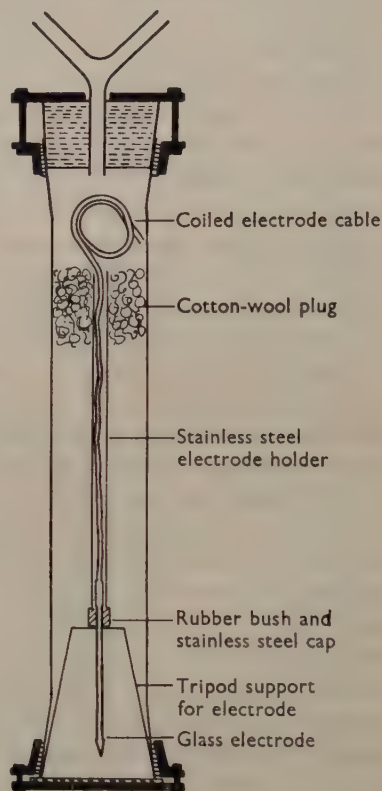


Fig. 3. Apparatus for sterilization of glass electrode by ethylene oxide gas.

amount of acid and alkali used can be measured. The lines from the reservoirs pass through solenoid-operated valves to a port on the reactor head. An arrangement of concentric tubes allows both lines to enter the reactor through the one port. This arrangement consists of a 30 cm. length of Pyrex glass capillary tube inside a second glass tube having minimal clearance. In most of the experiments reported the tubes terminated about 25 mm. above the liquid surface. Thus the rate of flow through the acid and alkali lines could be checked visually. Later, however, this was found to be unnecessary so that the ends of the concentric tubes could be placed below the medium surface. This was found to be advantageous (see below).

For the controlled addition of acid and alkali to the reactor a simple type of solenoid-operated tubing clamp (Fig. 2) has been used. It consists of a spring pressing on the silicone tubing of the acid or alkali line with sufficient force to provide a leak-free seal against the liquid head. When the solenoid is energized the arms of the plunger move forward on each side of the silicone tubing, lift the spring, and allow liquid to flow to the reactor. When the solenoid is de-energized the plunger is returned inside the coil by the spring. The solenoid valves require about 100 mA. to operate them and are energized directly from the 230 V. mains by the mercury switches of the controller. (The solenoids were supplied by Westool Ltd., St Helens, Auckland, Co. Durham, and designated series 500, model 5, rating 2 for 230 V. operation and were modified as shown in Fig. 2).

A commercial type of solenoid-operated pipe line valve has also been tried (magnetic valve, type R.B.G. seat diam. $\frac{3}{32}$ ins. for 230 V. a.c. operation, Teddington Industrial Equipment Ltd., Sunbury, Middlesex). This was used on the alkali line and gave completely satisfactory service for several hundred hours of operation. Eventually, however, the phosphor-bronze valve seat became corroded by the 0.5 N-alkali as expected and leakage occurred which became progressively worse. An all-stainless steel valve of similar type is now being obtained. For this type of valve the current consumption is higher than the permitted maximum (500 mA.) of the mercury switches of the pH controller. A relay (type F 103/3 for 230 V.a.c. operation, Sunvic Controls Ltd., 10 Essex Street, Strand, W.C. 2) was therefore interposed in this case. The time delay of the relay made no detectable reduction in the sensitivity of control.

The acid and alkali reservoirs and lines and the reactor are enclosed by shields of transparent Perspex sheet as a protection against possible breakage of the acid or alkali containers. For similar reasons all electrical control gear is grouped together on one panel fed from one 15 amp, 230 V. outlet outside the field of possible liquid contamination so that the entire electrical supply can be immediately cut off in the event of accident.

Acid and alkali for pH correction. 0.5 N- H_2SO_4 and 0.5 N-KOH were used at first. In continuous culture work, however, 1.5 N-alkali was used in order to decrease the volume of neutralizing material required.

Culture media. Two types of medium were used: a medium made from casein hydrolysate and yeast, and a defined medium consisting of glucose, ammonium sulphate, 0.2 % (w/v) KH_2PO_4 and traces of mineral salts.

Antifoam. The antifoam used was Alkaterge C diluted with liquid paraffin 1/10 and 3/10. Alkaterge C is a fatty acid derivative supplied by Commercial Solvents of Terre Haute, Indiana, U.S.A.

RESULTS

Sterilization of apparatus

The reactor without the glass electrode was sterilized in the autoclave. The end of the KCl bridge with the ceramic plug was sterilized *in situ* in the reactor. Some difficulty was experienced in completely filling the KCl bridge without

trapping air in the part within the reactor. It was later found that the best method of filling this part of the bridge was to sterilize it with a small temporary reservoir of KCl attached. During autoclaving the air was completely eliminated from the tube and replaced by KCl solution from the reservoir. After this the temporary reservoir was removed and connexion made to the proper reservoir, containing the reference electrode. Since the ceramic plug at the reactor end of the KCl bridge is a bacterial filter there is no necessity to sterilize the bridge system completely.

The acid and alkali supply units were sterilized separately. The KOH was sterilized in its reservoir. The sulphuric acid, in order to avoid any corrosion of the autoclave which might be caused by steam sterilization, was prepared by adding concentrated acid from a sterile pipette to the reservoir containing sterile distilled water. The acid and alkali lines were connected to the reactor with sterile precautions.

The glass electrodes available could not be sterilized by autoclaving because this caused failure of the insulation; some workers in America, it should be noted (e.g. Hosler & Johnson, 1953; Wheat, 1953), reported that they used steam-sterilized electrodes. Sterilization with liquid disinfectants is complicated and lengthy because means of protecting the cable and connexion from the disinfectant must be devised and washing with sterile water is necessary to remove the disinfectant. To overcome these difficulties, Neish & Ledingham (1949) used ultraviolet irradiation for sterilization of electrodes. This method too has its complications, for example, the need for crevice-free electrodes (see Watson, Clement & Muirhead, 1950). We decided to use ethylene oxide vapour as sterilizing agent for the glass electrode. Its main advantages are: (a) the whole electrode assembly including the cable can be immersed in the gas without causing corrosion or affecting the insulation; (b) on withdrawing from the gas the electrode may be easily freed from the sterilizing agent. The method adopted was to insert the electrode in its tube into a cylinder as shown in Fig. 3. The cylinder was evacuated by a water pump and filled with ethylene oxide gas and air in about equal proportions. The cylinder was then closed and left for 4 hr. at room temperature; 4 hr. were sufficient to ensure sterility but no experiments were made to find the minimum time required. After sterilization, the ethylene oxide vapour was largely displaced from the tube by evacuation and filling with sterile air three or four times. Finally the electrode was removed from the cylinder and inserted into the port on the reactor head. This operation was done against a stream of sterile air from the reactor to minimize the chance of contamination.

Standardization of pH meter with electrodes in the reactor

Two methods of standardizing the pH meter were used. The pH value of a sample of medium from the reactor was determined by another pH meter. The reactor pH meter was then set to read the found pH value of the medium. A second method dispensed with the need for two pH meters; a second set of electrodes only is required. The reactor pH meter was connected to a pair of electrodes outside the reactor and standardized, using buffer solutions. Using

the external electrodes the pH value of a sample of the medium in the reactor was determined. The reactor electrodes were then connected to the meter and the meter set to read the previously found pH value of the medium.

Evaluation of the pH control system

The installation has been subjected to comprehensive tests by using it to control the pH value of cultures of *Aerobacter aerogenes*. Two different culture media were used: (1) the casein hydrolysate and yeast medium in which without control the pH value rose during a culture; (2) the defined medium in which growth of the organism without control caused a decrease in pH value. The quantity of organism obtained in these cultures was 4–8 mg. dry wt./ml. The apparatus was operated continuously under growth conditions for periods of several hundred hours. This was done (a) by successive batch runs in which most of the old culture was removed daily and replaced by fresh medium, and (b) by using the system in continuous culture.

Figs. 4–6 show the results of various tests. Fig. 4a shows the stability of the pH meter reading in sterile casein hydrolysate + yeast medium over a period of 7 hr. Fig. 4b shows the drift of the pH value of a batch culture of the organism in the casein hydrolysate + yeast medium without control. The chart with the same medium and the pH controlled at pH 6.55 is shown in Fig. 4c; the exponential increase in the frequency of acid addition which paralleled the growth curve, is apparent.

The first pH-controlled run showed that it was necessary to limit the rates at which acid or alkali were delivered when the solenoid-operated valves were open. This was done partly by the use of some capillary tubing in the feed line and partly by an adjustable Hoffman screw clamp. A suitable flow-rate was found to be roughly 0.05 ml./sec. If this rate was much exceeded overshoot of the control point occurred and hunting of the controller resulted. Once the flow-rate of the acid and alkali had been limited in this way it was found that the control equipment was capable of maintaining a given pH value within ± 0.05 unit for very long periods provided that the culture pH tended to change only in one direction. If the change of pH value brought about by the organism changed from, say, a downward drift to an upward one the mean pH value was displaced by 0.09 pH unit, although the variation about the mean was still ± 0.05 unit; this point is illustrated in Fig. 5a. The reversal of the pH drift was effected by using a mixture of the two types of media. This change in the mean is due to the fact that in order to minimize operation of the solenoid valves and help to prevent hunting of the controller there is a small range of pH close to the control point within which neither solenoid valve is operated. The width of this neutral zone in the present instrument, preset by the manufacturers, is 0.08 pH unit.

The presence of a foam layer above the culture fluid acts as a barrier to the entry of acid or alkali if the inlet is above the liquid surface and thus decreases the sensitivity of the control system. This is clearly shown in Fig. 5b, where the width of the chart-trace increases as foam builds up and resumes normal limits after the addition of antifoam agent. The antifoam oil did not affect the

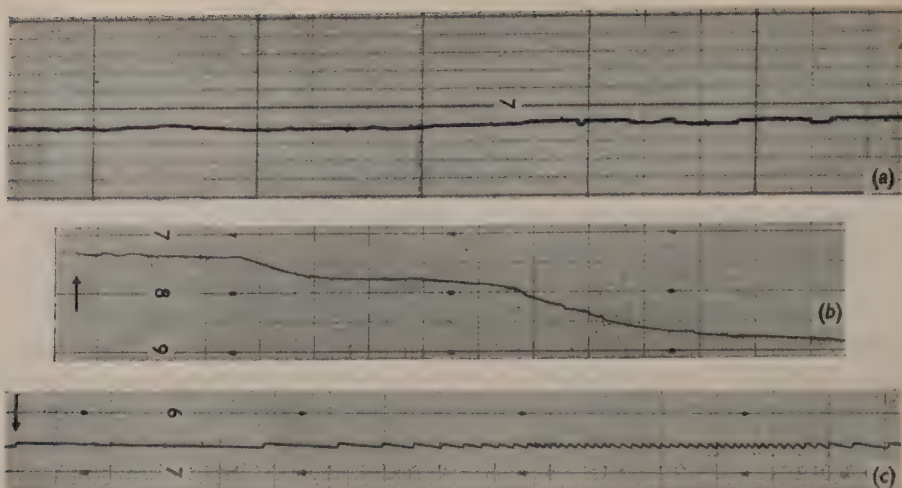


Fig. 4. In the three charts reproduced, the heavy vertical lines represent 30 min. intervals. The numbers are actual pH units so that one small horizontal division represents 0.1 unit. (a) Part of chart of 4-day stability test of Electrode/Meter system with sterile casein hydrolysate yeast medium in the reactor. (b) pH record of batch growth of *Aerobacter aerogenes* in casein + yeast medium without pH control. The arrow indicates point of inoculation. (c) pH record of batch growth of *A. aerogenes* in casein + yeast medium with automatic pH control by addition of 0.5 N-sulphuric acid. The pH value was set at 6.55. The arrow indicates point of inoculation.

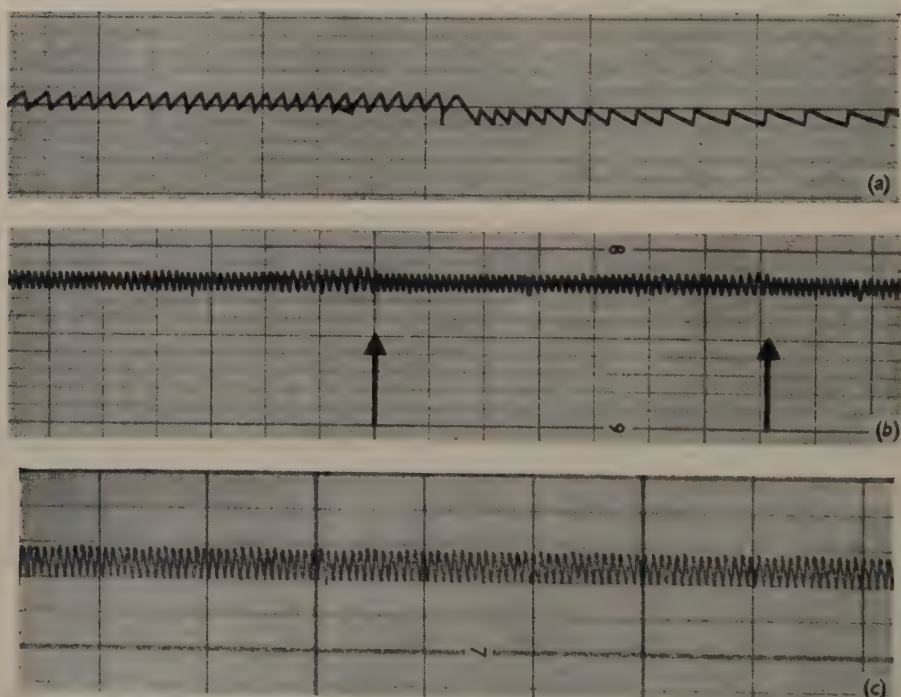


Fig. 5. In the charts reproduced the heavy vertical lines represent intervals of 1 hr. The numbers represent actual pH units. (a) Part of pH chart showing change-over from alkali addition to acid addition. (b) Part of pH chart showing the effect of foaming in the reactor on sensitivity of control. Arrows indicate where antifoam agent was added. (c) Part of pH chart showing extremely rapid working of control system to maintain a constant pH value against a continuous alkali leak.

electrodes in any way. This was shown by adding the antifoam in concentrations up to 10 ml./l. Complete elimination of interference by foaming was achieved by arranging for the point of entry of acid and alkali to be below the liquid surface.

As mentioned earlier, at one stage of the work a solenoid valve which was being tested became corroded and a continuous leak of alkali to the reactor occurred. Even under these conditions of heavy demand the pH controller maintained the pH value at the set point and indeed the control was better than ± 0.05 unit as shown in the enlarged tracing in Fig. 5c. These conditions were maintained for 18 hr. until the alkali valve was replaced.

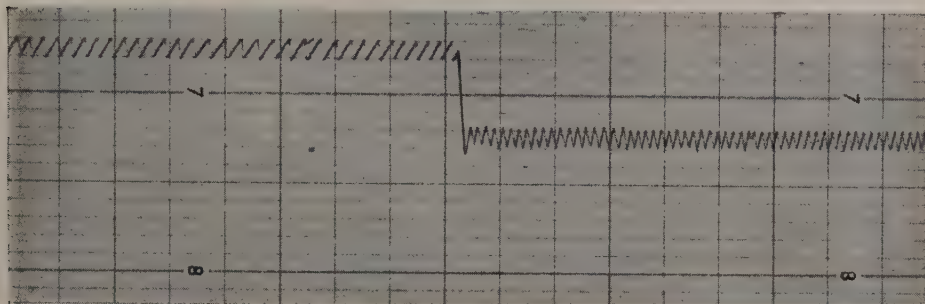


Fig. 6. Part of pH chart showing an alteration of the control point from pH 6.75 units to pH 7.24 units during a continuous culture. The incoming medium had a pH of 6.20. The heavy vertical lines represent intervals of 1 hr. The numbers represent actual pH units.

The smooth and rapid changeover from one pH value to another when desired is illustrated in Fig. 6. By means of the apparatus we have grown *Aerobacter aerogenes* in continuous culture at controlled pH values ranging from 4.75 to 8.25.

Over 2000 hr. of trouble-free operation of the pH control equipment have been obtained, mostly in continuous runs of 300–600 hr. In this period regular checks of the standardization of the reactor pH meter were made, and it was found that the maximum error was ± 0.02 pH unit in 24 hr. Whenever samples were taken from the reactor for analytical purposes their pH value was measured by another meter. In this way it was checked that the pH value indicated by the reactor electrodes was in fact the true pH value of the culture. In no case did the difference between the two meters exceed 0.05 pH unit.

Limits of precision of pH control

No attempt has been made to assess the limits of precision of pH control attainable with the particular combination of instruments and control gear available. As stated above, control within ± 0.05 pH unit was obtained in the reactor with the acid and alkali inlets just above the liquid surface. Still greater accuracy (± 0.03 pH unit) was attained by having the acid and alkali inlets below the liquid surface. Several independent variables impose limits

on the accuracy attainable, such as the sensitivity and stability of the pH meter/electrode system, the speed of response of the control instrument/control gear system, the positioning of electrodes and point of entry of the correcting fluid and the efficiency of mixing in the reactor. Ideally the operation of the acid or alkali valve, entry of acid or alkali into the reactor, and registering of the new pH value should be instantaneous. Some workers, for various reasons, have sited the electrodes outside the reactor. This may be done when a continuous flow system is used or by circulating the culture through a loop. This may lead to appreciable time lags in the operation of various parts of the system and there may also be insufficient mixing in the region of the electrodes. Wheat (1953), for instance, reported that the pH control system he used was much more trouble-free when the electrodes were inserted inside the culture vessel rather than in a loop with circulation of the medium. A discussion and mathematical treatment of time lags and other factors involved in precision of control have been given by Greer & Chaplin (1943).

Life of glass electrodes

The conditions of continuous culture are exacting for all of the control equipment used since not only must a precise control be achieved but it must be maintained for relatively long periods of time. Before the start of this work, no information was available as to what useful life could be expected of a glass pH electrode continuously immersed in culture fluid. During the work described in this report, three glass electrodes have been used. The first of these failed after about 300 hr. use, but examination by the manufacturers disclosed that the fault was in a piece of poor-quality cable used for the electrode lead and not in the electrode itself. The second electrode failed after a similar time period. Here the fault may have been due to deterioration of the glass membrane. Unfortunately, this electrode was broken when removing it from the reactor for testing, so a proper examination could not be made. The third glass electrode is still in use after about 1600 hr. made up of four separate runs. It appears, therefore, that under the conditions within the reactor a life of several hundred hours at least can be expected from an average commercial glass electrode. In any case the design of the present electrode assembly and reactor head permits the substitution of either of the electrodes or the KCl bridge without difficulty should it become necessary during a culture.

Acknowledgement is made to Mr G. H. Clement who constructed the solenoid-operated tubing clamp and to Dr D. Herbert who suggested the design. We should like to express our appreciation of the help given by Mr A. L. Dean in the installation and maintenance of the electronic instruments used.

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The Gonidial Stages in *Spirillum* spp. and *Vibrio* spp.

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SUMMARY: In twelve strains, comprising three species of saprophytic *Spirillum* and two of *Vibrio*, a life cycle was observed in which small gonidia, resembling very tiny bacteria, sometimes motile by means of polar flagella, were formed within enlarged mother-cells. After a period of reproduction in this phase, the gonidia became progressively enlarged to reconstitute the original form. In one species of *Spirillum*, the cycle was induced by the addition of penicillin to the medium, and in this and certain other respects was reminiscent of the L-cycle.

Reports of gonidial reproduction in bacteria have frequently been made. These fall into three categories: the L-cycle, which has frequently been described but is still not well understood; cycles in which ultramicroscopic units take part, for example in *Mycobacterium tuberculosis*, and which are even less well understood; and thirdly, the gonidial cycle in which the reproductive units appear to resemble very small bacteria, frequently motile by means of polar flagella, and are formed within mother-cells. Examples of the last class, which is the subject of study, may be found in the literature from quite an early date. However, only recently have convincingly illustrated accounts been available, especially of spirochaetes (Hampp, Scott & Wyckoff, 1948; DeLamater, Haanes, Wiggall & Pillsbury, 1951) and of the aerobic nitrogen-fixing bacteria, *Rhizobium* spp. (Bisset & Hale, 1951) and *Azotobacter* spp. (Bisset & Hale, 1953). Although the taxonomic borderline between spirilla and spirochaetes is not well-defined, it appears that a properly documented account of gonidial reproduction in the large saprophytic spiral bacteria, and in the shorter forms, usually described as vibrios, is not so far available, and it is the purpose of this paper to present such evidence, as well as to examine certain aspects of this phenomenon in which it resembles, or alternatively is distinct from, the L-cycle.

METHODS

Twelve strains of bacteria of spiral morphology were isolated by adding approximately 0.2 % (w/v) of 'Difco' peptone to 25 ml. pond-water in a Petri dish. This was left at room temperature (*c.* 25°) for 48 hr. and the surface layer removed by pipette, transferred to a sterile atomizer and sprayed over plates of the sodium acetate medium described by Pringsheim & Robinow (1947). Colonies recognizable under the plate microscope as consisting of spirilla were picked off and subcultured on the same medium. In the case of *Spirillum volutans* strains, which failed to grow on this medium, cultures were obtained by inoculating tubes of an infusion of wheat-grains and soil which had been steamed on three successive days (Pijper, 1949).

Impression preparations were made according to the method of Klieneberger-Nobel (1949). For electron microscopy cultures were grown on collodion membranes over the same medium (Hillier, Knaysi & Baker, 1948). These were examined at periods varying from 3 hr. to 5 days of incubation at 25°.

Preparations were shadowed with 40 % palladium/gold and were examined under a Metropolitan-Vickers electron microscope EM3, at 75 kV intensity.

RESULTS

According to the diagnoses of Giesberger (1936), Myers (1940) and *Bergey's Manual* (1948) the bacteria isolated were classified as *Spirillum serpens* (4 strains), *S. undula* (6 strains), *S. volutans* (2 strains). In addition, an undiagnosed strain of *Vibrio*, isolated from pond-water, was examined. All these showed the gonidial life cycle. In addition, several strains of spiral bacteria were isolated having the characters of *Spirillum*, *Vibrio* and *Caulobacter* spp., in which this type of life cycle was not observed.

Spirillum undula. The entire cycle was usually complete in about 5 days. The normal form of the spirillum was approximately 1 μ . in diameter and from 20 or 30 μ . to as much as 100 μ . in length. Most frequently the organisms had 2½ to 3 complete coils, and possessed 20–30 flagellar fibrils at each pole (Pl. 1, figs. 1, 4). The beginning of the gonidial cycle was marked by the appearance of swellings, from one to three, but seldom more in number, along the length of the spirillum. From their first appearance, these swellings were seen to contain numerous small granules (Pl. 1, figs. 2–5). The swellings and the granules which they contained increased in size at the apparent expense of the spirillum (Pl. 1, figs. 4, 5), until the latter had almost disappeared. The swellings first appeared after 48 hr. of incubation, and the initial process was complete usually at the conclusion of a further 24 hr. Thereafter, the granules became freed from the mother-cells, in the form of tiny, slightly curved bacteria, actively motile by means of polar flagella (Pl. 2, fig. 6). These gonidia appeared to reproduce by fission. They also grew considerably in size, and by the end of 2–3 days were restored to the original condition of the parent organism. The entire cycle was concluded in a single culture. Of considerable interest was the clear demonstration of blepharoplasts in the gonidia.

Spirillum volutans. This species, considerably larger in size than the preceding one, developed in exactly the same manner, as far as could be determined.

Spirillum serpens. Cultures of this species, which morphologically resembled *S. undula*, grew for periods up to 5 weeks without producing gonidial forms. But it was found that these could be induced at any stage by the addition of 200 units penicillin/ml. to the culture medium (cf. production of L-forms under the influence of penicillin; Pierce, 1942; Klieneberger-Nobel, 1949; Dienes & Weinberger, 1951)). This process occurred very rapidly, and all stages were visible in a single culture within a few hours of the addition of penicillin. But the cysts did not germinate unless subcultured upon fresh medium

containing penicillin, when the granules increased further in size and appeared as small cocco-bacillary elements, devoid of flagella. These developed as tiny colonies reminiscent of the G-form colonies described by Hadley (1931) (Pl. 2, figs. 7, 8). On subculture upon medium without penicillin these developed into normal spirilla. When, in the process of this cycle, subculture was made at any stage upon medium which did not contain penicillin, the normal forms immediately outgrew the gonidia, so that further development of the latter was obscured (Pl. 2, fig. 9).

In addition, strains of *Spirillum serpens* produced organelles resembling motile gonidia by an alternative method. A small bud became detached, apparently by constriction, from the tip of the vegetative spirillum, retaining all the flagella appertaining to that pole; and this small, but highly motile cell proceeded independently (Pl. 3, figs. 10-12). The subsequent fate of these units was not determined.

Vibrio sp. A series of isolates from water taken from a small pond in the vicinity of the laboratory proved to be a small unidentifiable vibrio, about $0.5\ \mu$. in diameter (Pl. 4, fig. 13). This organism underwent a type of life cycle similar to that of *Spirillum serpens*, but initially did so spontaneously. On repeated subculture, however, it was found that the phenomenon appeared only on the addition of 1000 units penicillin/ml. to the medium. The cycle is illustrated in Pl. 4, figs 13-15.

Vibrio metchnikovii. For comparison with the last-mentioned species, a strain of this pathogenic vibrio was obtained from the National Collection of Type Cultures. In contrast, to the preceding *Vibrio* sp., *V. metchnikovii* produced motile gonidia with one or two flagella, very strongly resembling those of *Rhizobium* (Pl. 4, fig. 16).

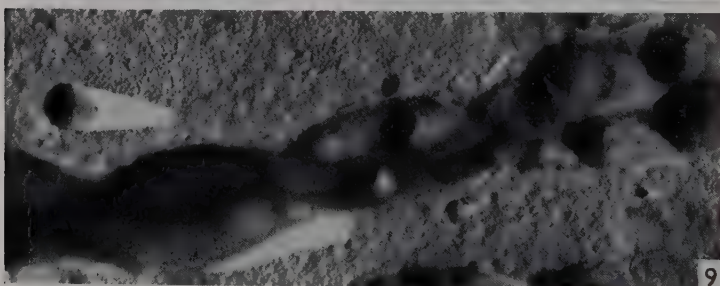
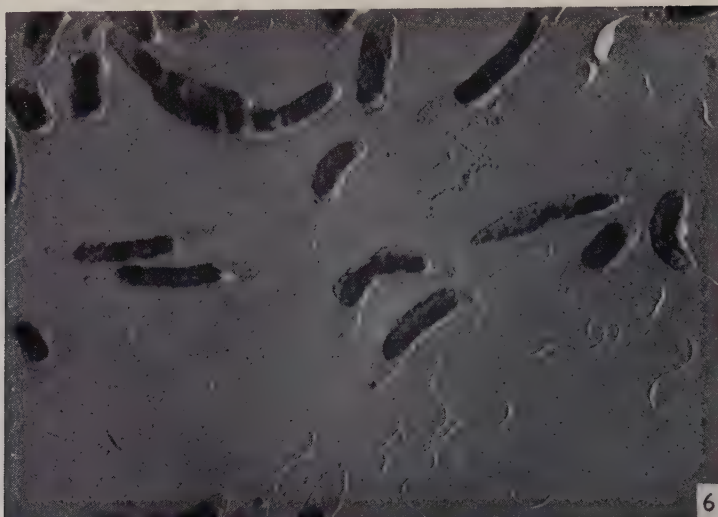
DISCUSSION

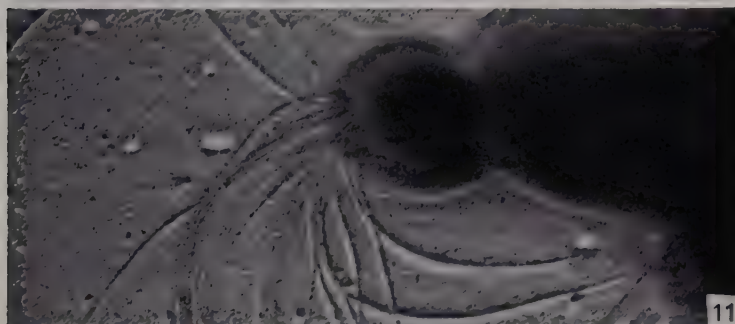
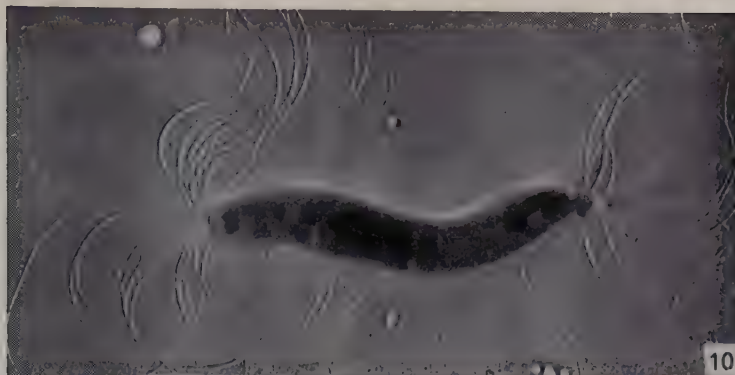
The gonidial cycle, both in the large saprophytic spirilla, and in species of *Vibrio*, appears in two similar but distinct forms. One of these is in all respects the same as the production of 'swarmers' in the nitrogen-fixing bacteria (Bisset & Hale, 1951, 1953). The reproductive elements are complete tiny bacteria with polar flagella, which reproduce as such but gradually grow into typical parent forms. In other species, however, the gonidia were not motile, and appeared most readily under the influence of penicillin. They manifested themselves as tiny colonies, and had therefore a certain amount in common both with the more perfect types of gonidia and with the so-called G- and L-forms. This condition may reasonably be considered to be in some respects degenerate, and it is of considerable interest that one species, *Spirillum serpens*, which had thus apparently lost the power of producing motile gonidia by this method, had evolved the system of budding-off motile elements from the tip of the vegetative spirillum. The existence of these tiny reproductive forms in the spiral bacteria serves to explain the phenomenon, well known to those who have worked with them, of sudden, mysterious fluctuations in the populations of these bacteria, both in nature and in artificial culture. It also provides evidence of similarity between the large spirilla and the spirochaetes, where such cycles have been long known.

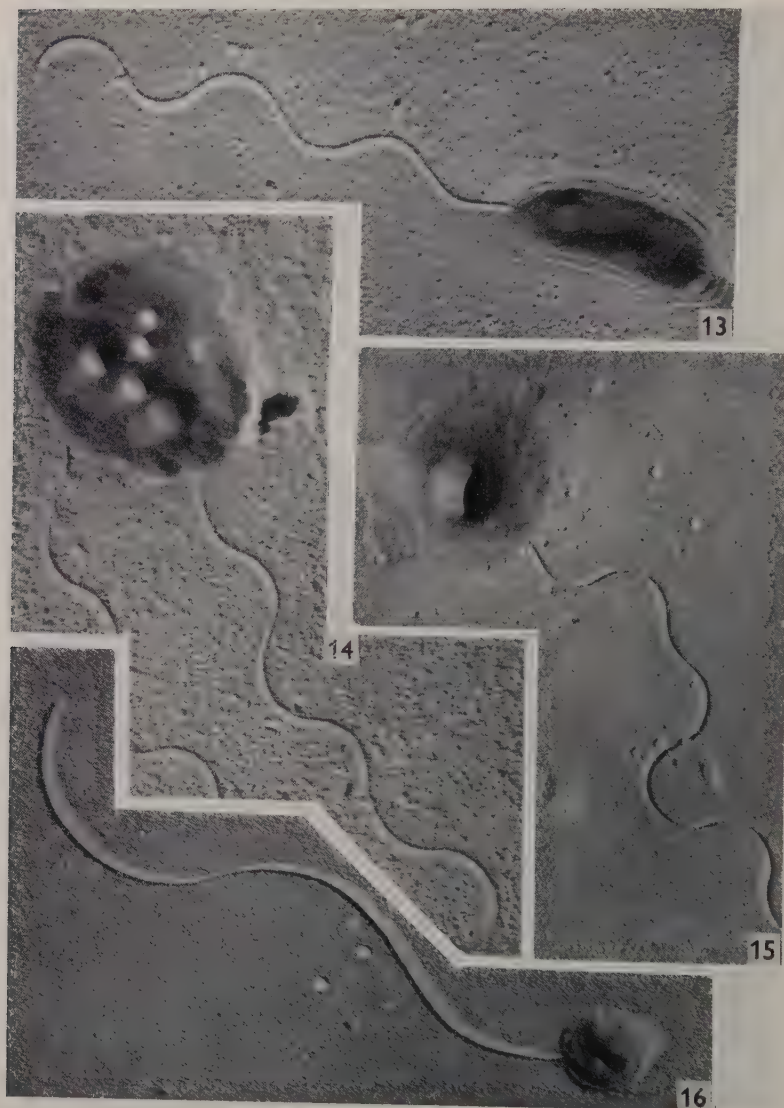


P. PEASE—GONIDIAL STAGES IN BACTERIA. PLATE 1

(Facing p. 674)







P. PEASE—GONIDIAL STAGES IN BACTERIA. PLATE 4

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. *Spirillum undula*. Flagellar fibrils arising from the pole of the organism. Electron micrograph, $\times 10,000$.
- Figs. 2, 3. *S. undula*. Granular structures within a colony. Impression preparations, $\times 1000$.
- Fig. 4. *S. undula*. Granules within an organism. Electron micrograph, $\times 6000$.
- Fig. 5. *S. undula*. Cyst containing granules. The remains of the organism can be seen still attached. Electron micrograph, $\times 6000$.

PLATE 2

- Fig. 6. *Spirillum undula*. Gonidia with single flagella attached to blepharoplasts. Electron micrograph, $\times 3000$.
- Figs. 7, 8. *S. serpens*. Colonies of cocco-bacillary elements. Electron micrographs, $\times 20,000$ and $10,000$.
- Fig. 9. *S. serpens*. Reversion of elements to spiral form. Electron micrograph, $\times 12,000$.

PLATE 3

- Figs. 10, 11. *Spirillum serpens*. Formation of a bud at the tip of an organism. Electron micrographs, $\times 6000$ and $28,000$.
- Fig. 12. *S. serpens*. A free bud. Electron micrograph, $\times 20,000$.

PLATE 4

- Fig. 13. Saprophytic *Vibrio* sp. with one polar flagellum. Electron micrograph, $\times 15,000$.
- Figs. 14, 15. Saprophytic *Vibrio* sp. containing granules. Electron micrographs, $\times 20,000$ and $\times 15,000$.
- Fig. 16. *Vibrio metchnikovii*. Motile gonidium. Electron micrograph, $\times 25,000$.

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A New Vi-phage Type of *Salmonella typhi*; with a Discussion of Methods of Preparation of Typing Phages for New Vi-Types

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SUMMARY: A new Vi-phage type of *Salmonella typhi*, T4904, is described. The homologous typing phage (phage 4904) was prepared from phage D6, a host-range mutant of Vi-phage II. The new type belongs to the E group and consists of type E1 carrying the determining phage d6. The formula E1(d6) has therefore been assigned to it. The host range of phage 4904, the homologous typing phage, is considered in relation to the structural formula of T4904. This formula fully explains the ability of the phage to lyse strains other than T4904. The general aspects of adaptation of Vi-phage II to new Vi-types of *S. typhi* are discussed. It is shown that the older attitudes to this subject require modification in the light of recent work. The applications of the results of study of the temperate type-determining phages to the solution of practical problems in Vi-phage typing are described.

The commoner Vi-phage types of *Salmonella typhi* were described some years ago (Craigie & Yen, 1938; Craigie & Felix, 1947), but strains which represent new, rather rare, types are still encountered occasionally. The type to be described was isolated in October 1952 from the blood and faeces of a Chinese girl of 18 years who was suffering from clinical enteric fever; the strain was numbered T4904. The patient lived with her brother in Bradford, and on investigation the brother, who was free from symptoms, was found to be excreting *S. typhi* in his urine. He was 25 years of age and had come to this country from Hong Kong in 1949. No history of typhoid fever was obtained from this man, but it was assumed that he was a chronic carrier. The strain isolated from him proved to belong to the same Vi-type as T4904. It is not yet known whether this type is endemic to Hong Kong. Reference has been made to T4904 in a previous publication (Anderson & Fraser, 1955*a*) but a full description of the new type seems worthwhile because the work on it provided opportunities for the practical confirmation of hypotheses recently advanced by the author (Anderson, 1955; Anderson & Fraser, 1955*a*).

The Vi-types of *Salmonella typhi* and their corresponding typing phages, all of the latter being derived from Vi-phage II of Craigie & Yen (1938), are designated by identical symbols. For example, type D6 is lysed by phage D6. A number of Vi-types of *S. typhi* owe their type specificity in part to the presence of temperate phages which are designated 'type-determining' phages (Anderson, 1951; Felix & Anderson, 1951; Anderson & Felix, 1953*b*). The remainder of the specificity is controlled by the non-lysogenic precursors of the types. The term 'non-lysogenic' is used only in relation to the absence of type-determining phages. Many temperate phages that do not have a type-

determining function are found in *S. typhi*; the presence or absence of these is not considered here. The type-determining phages are designated by small letters corresponding to the capitals used for the respective lysogenic Vi-types, and by a number with a superscript prime sign when the Vi-type from which they are isolated is designated numerically. Thus, type D6 carries phage d6 and type 25 phage 25'. The determining phages are unrelated to Vi-phage II, from which the typing phages are derived. The recent suggestions concerning the use of structural formulae for the lysogenically determined Vi-types (Anderson, 1955; Anderson & Fraser, 1955*a*) will be adopted at relevant points in this paper. The formulae consist of the symbol of the non-lysogenic precursor type followed by that of the temperate type-determining phage in parenthesis. Thus, type D6, which can be prepared artificially by lysogenizing type A with phage d6, is designated A(d6). Although they appear to be distinct, phages f2 and 30' possess identical type-determining characters and one only may be specified in the formulae of types which may be determined by either. For example, type 29 can have the alternative formulae of A(f2) or A(30'); for convenience, only one of these formulae may be given.

EXPERIMENTAL

Media

Bacto dehydrated nutrient broth (Difco Laboratories), in a strength of 2%, was the nutrient basis of all media used in the experiments described in this paper. For the preparation of solid media 1.3% New Zealand powdered agar was added.

Examination of T 4904

Tests with the Vi-typing phages showed that T 4904 was resistant to all the available 'adapted' preparations of Vi-phage II in routine test dilution (R.T.D.). Two other Vi-phages described by Craigie & Yen (1938) are used in this laboratory in routine typing tests. These are known as Vi-phages I and IV. T 4904 was sensitive to the former but not to the latter of these phages. It was resistant to a non-Vi phage used in this laboratory in routine phage-typing tests.*

Phage A is probably the wild type of Vi-phage II (Anderson & Fraser, 1955*a*) and has been regarded as the most suitable starting-point for the preparation of typing phages for new Vi-types (Craigie & Felix, 1947; Anderson & Felix, 1953*a*). Attempts were made, therefore, to adapt phage A to T 4904, but these were unsuccessful. Although the strain was resistant to the routine typing adaptations of Vi-phage II, that is, no considerable degree of lysis occurred with any typing phage, phage D6 regularly produced a few plaques

* This phage was mentioned originally by Felix & Callow (1943) and has been previously referred to as an 'O phage' (see, for instance, Anderson & Felix, 1953*a*). Unpublished observations made by the present writer in 1949 showed that this phage attacks the majority of salmonellas and, in view of this indication of non-specificity in regard to the O complex, and also because it attacks some rough salmonellas lacking the O antigen, the designation 'non-Vi phage' is most suitable.

on T4904 and phage E1 occasionally did the same. A detailed titration of phage D6 on type A and T4904 revealed that its titre on T4904 was 1/1,000 of its titre on type A. Single plaques were cut from agar plates in which phage D6 had been titrated on T4904, together with a small amount of the surrounding culture, transferred to 20 ml. amounts of nutrient broth in screw-capped bottles, and incubated for 5 hr. at 38.5°. The resulting lysates were heated to 57° for 40 min. to kill the host cells, centrifuged to remove the dead bacilli, and titrated on types A and D6 and on T4904. This titration showed that the newly adapted phages had titres approaching 10^{10} , that they attacked all three of the indicator strains used equally well, and that they had R.T.D.'s of at least 1/5000. If they exhibited a satisfactory degree of specificity, therefore, they would be suitable for use as typing phages for the new type represented by T4904.

A single line of the adapted phage, which will be designated hereafter 'phage 4904', was chosen for further scrutiny. Since its preparation in 1952 this phage has been tested on a number of occasions on all the available Vi-type strains of *Salmonella typhi*, and T 4904 has similarly been subjected to many tests with all the available typing phages. The results of this work can be summarized as follows:

(1) Phage 4904 in R.T.D. produces the same degree of lysis on types A, D1, D5, D6, E1, 29 and T4904. It also lyses equally well type E7, which was first prepared artificially and defined by the author in 1951 (see Felix & Anderson, 1951; Anderson & Felix, 1953*b*) and has recently been discovered in the field by Scholtens. The designation type E7 is recent (see Scholtens, 1955). (The author's type E7 has the formula E1(f2), whereas Scholtens's representative of the type appears to be E1(30'); for a further discussion of this subject see Anderson & Fraser, 1955*a*). Phage 4904 does not attack the remaining specific Vi-types of *Salmonella typhi*. It is neutralized by anti-Vi-phage II serum prepared by immunizing rabbits with phage A.

(2) T4904 is fully lysed only by its homologous adapted phage in R.T.D. It is also sensitive to a lesser extent to phage E7.

These findings prove that T4904 represents a new type. The cross-reactions of its homologous typing phage will be discussed later.

The structure of the new type

T4904 was found to be carrying a temperate phage which was destroyed by heating to 57° for 30 min., formed micro-plaques on types A, C and E1 and did not attack types D6, F2, 29, 30 or T4904. Type A, when lysogenized with this phage, became type D6; the phage converted type C into type C2 (which is also known as type 33), and type E1 into a type identical with that of T4904. The changes undergone by types A and C in this series of experiments showed that the phage carried by T4904 belongs to the d6, f2, 30' group of type-determining phages, and it appears to be indistinguishable from phage d6 described in previous publications (Felix & Anderson, 1951; Anderson & Felix, 1953*b*).

Strains carrying phages of the d6, f2, 30' group tend on storage to lose their

determining phage so that non-lysogenic variants can be isolated from them (Anderson, 1951; Felix & Anderson, 1951). When T4904 was stored at room temperature on dry Dorset egg slopes without subculture for about a year it exhibited greatly increased cross-reactions with phages E1 and E2, and a number of single-colony isolations from such a culture proved to belong to type E1 (which is fully sensitive to phages E1 and E2) and were non-lysogenic. These E1 lines, when lysogenized with phage d6 or with the determining phage carried by T4904, were re-converted into strains showing phage sensitivities identical with those of T4904. This work proved that T4904 consists of type E1 carrying phage d6: it thus belongs to the E group of Vi-types and the structural formula E1(d6) has been assigned to it in accordance with the suggestions of Anderson (1955) and Anderson & Fraser (1955*a*).

The host range of phage 4904

It has been pointed out that the host range of phage 4904 includes types A, D1, D5, D6, E1, E7 and 29 as well as the homologous strain. Type A is lysed by all adaptations of Vi-phage II and can be omitted from the following discussion. The remainder of the host range of phage 4904 can be explained on the principles recently described by Anderson (1955) and Anderson & Fraser (1955*a*). T4904 carries the determining phage d6. It can only be lysed, therefore, by the host-range mutant of Vi-phage II which is able to overcome the obstacle offered by this determining phage; this mutant is the same as that which can lyse type D6 (structural formula A(d6)) and, when obtained in the pure state, constitutes the typing phage D6. Therefore, the typing phage for T4904 will lyse type D6 and any other types lysed by phage D6. Such types are, in the D group, D1 and D5. The ability of phage 4904 to lyse type E1 is due to the fact that T4904 has the formula E1(d6) and, in adaptation to it, Vi-phage II undergoes a phenotypic change identical with that elicited by type E1. Phages d6, f2 and 30' are closely similar in properties (see Anderson & Felix, 1953*a*), but strains carrying phage d6 are relatively resistant to Vi-phage II adapted to strains carrying phages f2 and 30'. The converse does not hold, however, for strains carrying phages f2 and 30' are fully sensitive to adaptations of Vi-phage II to strains carrying phage d6. Thus, type 29 (= A(f2) or A(30')) is fully sensitive to phage 4904 because the latter is adapted to a strain of the formula E1(d6). Moreover, as phage 4904 possesses the E1 adaptation, it will also lyse type E7, which has the alternative formulae of E1(f2) or E1(30'), as effectively as it lyses type 29.

DISCUSSION

There has been a tendency to exaggerate the importance of using phage A as the starting phage for the adaptation of Craigie & Yen's Vi-phage II to new Vi-types of the typhoid bacillus (see, for example, Craigie & Felix, 1947; Anderson & Felix, 1953*a*). This has been due to lack of knowledge of the processes underlying the phenomena of adaptation. It was shown by Anderson & Felix (1952, 1953*a*, *c*) that both phenotypic and genotypic changes played

a part in the evolution of the Vi-typing phages. However, it was suggested by the same authors (1953*a, c*) that the whole process of adaptation of Vi-phage II to *Salmonella typhi* was phenotypic in nature. This suggestion would be confirmed if a host cell were identified which could precipitate the total reversion of host range of apparently permanently altered preparations to that of phage A, which is probably the wild type of Vi-phage II. Recent work has shown this view to be incorrect (Anderson, 1955; Anderson & Fraser, 1955*a, b*). It has been shown that Vi-typing phages having stable host ranges which are different from that of phage A are host-range mutants of Vi-phage II. They exhibit a clonal distribution in fluctuation tests and pre-exist in concentrated stocks of phage A. Thus, the adaptation of phage A to Vi-types of *S. typhi* that can only be lysed by such host-range mutants is a process of selection of the mutants concerned.

Determination of the genotype of Vi-typing phages

The genotype of a typing adaptation of Vi-phage II can be determined by growing the phage on type A. Under such conditions the phenotypically modifiable portion of the phage reverts to the wild state, thus unmasking the basic genotype. The change of an adapted phage to phage A when grown on type A indicates that it had undergone a phenotypic change only during its original adaptation, and that it possesses the wild genotype of Vi-phage II. As the majority of the lysogenically determined types can be lysed only by host-range mutants of Vi-phage II (Anderson & Fraser, 1955*a*), the reversion of an adapted phage to phage A by growth on type A suggests that the Vi-type of *Salmonella typhi* responsible for the earlier adaptation is not lysogenically determined. Thus, phage E1 changes to phage A during propagation on Type A. As phage A is the wild genotype of Vi-phage II the change undergone by phage E1 indicates that it is unlikely that type E1 owes its type specificity to the presence of a determining phage; no determining phage has hitherto been isolated from type E1. In contrast to the foregoing example is that of phages F2, 30 and E7 which correspond to Vi-types of *S. typhi* having the formulae F1(f2), C(f2) and E1(f2), respectively. They all attack types A and 29 (type 29 has the formula A(f2)) equally well. When these phages are grown on type A they all change to a phage corresponding to a Vi-type of the formula A(f2), that is, to phage 29. This phage lyses only types A and 29 and represents the basic host-range mutant which is capable of overcoming the block in the multiplication of the wild genotype of Vi-phage II produced by the presence of the determining phage f2. It will be seen that an experiment of the type just described gives valuable information concerning the nature of the determining phage that may be carried by a Vi-type of which the structural formula is unknown.

These observations necessitate a change of attitude towards the adaptation of Vi-phage II to types of *Salmonella typhi* that can only be lysed by host-range mutants of the Vi-phage. Many such types owe their specificity to determining phages. T4904 provides an excellent example of this phenomenon. As its formula is E1(d6) it demands two changes in Vi-phage II before it will support

the growth of this phage. Firstly, there is the host-range mutation which enables the Vi phage to overcome the barrier to its multiplication erected by phage d6; this mutant occurs in concentrated populations of phage A with a frequency of about 10^{-6} and, as has been indicated above, is identical with the typing phage D6. Secondly, the selected mutant particle must undergo phenotypic modification to enable it to multiply in the E1 component of the E1(d6) complex; such modifications occur with a frequency of about 10^{-3} . Thus, when a concentrated stock of phage A is applied to a culture of the formula E1(d6) the frequency with which the necessary double event will enable a suitable particle to encounter a cell that it can lyse without obstruction is of the minute order of 10^{-9} . The mutant selected will have the same genotype as that which lyses type D6 (=A(d6)), that is, it will have the genotype of phage D6. The slender chance of detecting such an occurrence is complicated by the possibility that the mutant selected by strain E1(d6) from the parent population of phage A may differ in some details of host range from that of the known phage D6; this will cause confusion when its host range is examined and when the relations of its homologous type strain to other types are being defined.* A rational approach, therefore, when attempting to adapt Vi-phage II to a strain with a formula such as E1(d6), is to commence with a mutant of correct initial host range when possible; the chances of successful adaptation thereby become greatly improved. For example, the adaptation of phage D6 to E1(d6) requires only the phenotypic change to cover the E1 specificity; the frequency of this change is 10^{-3} —an enormous improvement on the 10^{-9} chance indicated above.

The adaptation of Vi-phage II to new types of Salmonella typhi

The practical application of these principles concerns the adaptation of Vi-phage II to new types that carry determining phages. Not all types are lysogenically determined, and a number of apparently non-lysogenic types can be lysed only by host-range mutants of Vi-phage II. In such cases no information at present available suggests that other preparations of Vi-phage II than phage A might be more suitably used as the starting-point for the new typing phages. Naturally, as phage A seems to represent the wild phenotype and genotype of Vi-phage II, it is on general grounds the most satisfactory phage to use initially in attempting adaptations to new types. But if difficulty is experienced in adapting phage A to a new type, attempts should be made to isolate and identify a determining phage from the new type. T4904 can again be used as an example: had attempts to prepare a typing phage for it been confined to phage A, the new type might not have been identified. However,

* Such a situation arose in connexion with the adaptation of Vi-phage II to type C2(=C(d6)). Desranleau, who discovered the type (see Desranleau & Martin, 1950), selected a mutant of Vi-phage II that lyses type D5 very poorly. On the other hand, Dr P. R. Edwards of the Communicable Diseases Center, Chamblee, Georgia, U.S.A., who adapted Vi-phage II to type C2 independently, selected the true D6 mutant which lyses type D5. We have also demonstrated that C2 can select a mutant having the genotype of phage 29 (type 29=A(f2)); this lyses strains carrying phage d6 less readily than does the D6 mutant.

a determining phage could have been isolated from the strain before its Vi-type was established. The lysogenization of type A with this phage would yield type D6 (=A(d6)), and the identity of the determining phage would thereby be established as phage d6. It would then have been evident that the most satisfactory method of preparing a typing phage for T 4904 was to start with phage D6, thus by-passing the step which involves the uncertainty of selecting this host-range mutant from phage A.

An indication has been given in a previous publication (Anderson & Fraser, 1955*a*) of how the investigation of type-determining phages can help in the preparation of typing phages for Vi-types that have already been defined. The preceding discussion shows that the method could be applied equally well to the preparation of typing phages for lysogenically determined types that have not yet been identified.

It has been suggested (Anderson, 1955; Anderson & Fraser, 1955*a*) that, for the study of the phenomena underlying the Vi-phage typing method, the use of structural formulae for the lysogenically determined types, and the use of a system of grouping of lysogenic types based on non-lysogenic ancestors possessed in common by certain types, afford considerable help. This can be seen from the work presented above. The types that do not owe their specificity to determining phages remain enigmata at present, but much information is now available about the lysogenically determined types and their respective typing phages. The identification of the determining phage carried by T 4904, and of the non-lysogenic precursor of the type, furnishes us with a structural formula for the new type. By the application of the principles outlined recently (Anderson, 1955; Anderson & Fraser, 1955*a*), it is possible from this formula to deduce the host range of the preparation of Vi-phage II adapted to the type. The formula is E1(d6). The homologous typing preparation is a phenotypically modified mutant of Vi-phage II that, as has been explained earlier, can be expected to attack type D6 (=A(d6)), type E1, type E7 (=E1(f2) or E1(30')) and type 29 (=A(f2) or A(30')) as well as the homologous type. In addition, types D1 and D5 will be lysed by phage 4904 because they are sensitive to phage D6. The formula of T 4904 thus fully explains why its adapted typing phage shows cross-reactions in the D and E groups of Vi-types. Finally, a logical reason is provided for placing T 4904 in the E group of Vi-types because its non-lysogenic component is type E1, and grouping is preferably based on the nature of non-lysogenic precursor types.

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A Study of the Metabolism of Phenolic Compounds by Soil Fungi Using Spore Suspensions

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SUMMARY: Spore suspensions were used to investigate the metabolism of *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin by *Haplographium* sp., *Hormodendrum* sp., *Penicillium* sp. and *Spicaria* sp. The intermediate products of their metabolism, *p*-hydroxybenzoic, syringic and vanillic acids, were found to be attacked by adaptive enzymes. The formation of these enzymes was greatly decreased by the antibiotic citrinin.

The results of experiments, based on a technique in which fungal spore suspensions were used in place of hyphae, are presented in this paper. Under investigation was the metabolism of certain phenolic compounds related to lignin, which have already been reported to be utilized by soil microfungi as sources of carbon (Henderson & Farmer, 1955).

METHODS

Organisms. The following fungi were used in this investigation: *Haplographium* sp. from deciduous woodland soil; *Hormodendrum* sp. no. 1 from sand under Scots Pine in an afforested sand dune; *Penicillium* sp. no. 13 from moorland soil; *Spicaria* sp. from foreshore sand. These fungi were all used in the previous investigation (Henderson & Farmer, 1955).

Preparation of spore suspensions. The method used was that previously described (Henderson, 1955). Spores were obtained from cultures of the fungi grown on potato glucose agar for 7 days at 22°. The fungi were grown on strips of cellophan which were laid on the surface of the agar and which were inoculated by streaking with spores. The strips, bearing the growth, were transferred to tubes containing a solution of Tween 80 (1/1000, v/v) in which the spores suspended uniformly. On shaking gently the spores became detached while the mycelium adhered to the cellophan. The spores were centrifuged, washed twice in distilled water and finally suspended in distilled water. All operations were carried out aseptically. The density of the suspensions was measured by means of a haemocytometer.

Solutions added to Warburg flasks. The flasks and all solutions, with the exception of KOH, were sterilized by autoclaving or by filtration in the case of the phenolic compounds, and all operations were carried out aseptically. Solutions added to the main compartment were: 0.5 ml. mineral salts; 0.5 ml. Difco yeast extract; 0.5 ml. buffer and 1.0 ml. spore suspension. 0.5 ml. substrate was added to the side arm and 0.2 ml. 5% (w/v) KOH to the centre cup. The mineral salts solution was a modified Czapek mineral salts solution, as used in the growth experiments (Henderson & Farmer, 1955). It contained: NaNO_3 , 1.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g.; KH_2PO_4 , 0.5 g.; KCl, 0.25 g. dissolved in 500 ml. water. The pH value and kind of buffer used for each organism was

determined by preliminary experiments (see later). The spores were incubated overnight in the Warburg vessels before addition of substrates and the concentration of yeast extract present was such that it stimulated germination but did not support too high a basal rate of respiration on the following day. The concentration of yeast extract finally used for *Haplographium* sp., *Penicillium* sp. and *Spicaria* sp. was 0.125 % (w/v) and for *Hormodendrum* sp. 0.48 % (w/v).

The experiments were conducted at 25° in air. Following the overnight incubation, readings were taken at 30 or 60 min. intervals for 1–2 hr. to establish the basal respiration rate. Substrates were then tipped in and oxygen uptake measurements were continued at 30 or 60 min. intervals for the duration of the experiments, which was generally 7–8 hr.

RESULTS

Influence of pH value on the respiration rates of the fungi

Experiments, which were designed to find the optimum pH value for each fungus, revealed that the respiratory rates were highest at pH values which corresponded very closely with those of the various 'soils' from which the fungi were isolated. McIlvaine's citrate + phosphate buffer (Clark, 1928) was used at pH 3, 5 and 7 with each fungus. In these experiments no source of carbon was added in addition to that present in the yeast extract.

Penicillium sp., which showed a distinct optimum at pH 3, was isolated from raw heather humus, the pH value of which was 3.5. *Spicaria* sp. respired more rapidly at pH 5 and 7 than at pH 3. This species was isolated from foreshore sand of pH 7.3. *Hormodendrum* sp., isolated from sand under Scots Pine of pH 4.2, showed a distinct optimum at pH 5 with McIlvaine's buffer. However, as this species was found to oxidize citric acid, Sørensen's inorganic phosphate buffer (Clark, 1928) was used at pH 5.3, which is the lowest value of its range, for this fungus. Sørensen's buffer at pH 5.3 was also used for *Haplographium* sp. which was isolated from deciduous woodland soil of pH 5.7.

Respiration in the presence of phenolic compounds

In these experiments 0.5 ml. of 0.1 % (w/v) solutions of *p*-hydroxybenzaldehyde, syringaldehyde or vanillin or of 0.05 % (w/v) ferulic acid were tipped into the vessels from the side arms after the overnight incubation period. The results obtained from experiments with the four fungi (see Table 1) resembled closely those previously obtained from growth experiments (Henderson & Farmer, 1955). The latter experiments indicated that *p*-hydroxybenzaldehyde was the most readily utilizable compound and none of the above species left any residue of it in the culture media. Similarly, all four gave rapid oxygen uptakes on the addition of *p*-hydroxybenzaldehyde to spore suspensions. None of these species left any vanillin unaltered in the growth experiments, and the addition of this substance to spore suspensions gave rise to increased oxygen uptakes. In growth experiments referred to above, *Haplographium* sp., *Penicillium* sp. and *Spicaria* sp. all left residues of syringaldehyde which in the

case of the first two amounted to more than half of that added originally. *Hormodendrum* sp. removed it entirely by the end of the growth period, but it was more slowly attacked by this fungus than were the other three compounds. The slower rate of attack on this compound was confirmed by the respiration studies. *Haplographium* sp., *Penicillium* sp. and *Spicaria* sp. gave a small

Table 1. *Respiration of Haplographium sp., Hormodendrum sp., Penicillium sp. and Spicaria sp. on p-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin*

1 ml. of spore suspension added to vessels (*Haplographium* 3.1×10^9 ; *Hormodendrum* 9.3×10^8 ; *Penicillium*?; *Spicaria* 1.2×10^9 spores/ml.) + 0.5 ml. mineral salts + 0.5 ml. buffer (*Haplographium* and *Hormodendrum* Sørensen's phosphate buffer pH 5.3; *Penicillium* McIlvaine's citrate-phosphate buffer pH 3.0; *Spicaria* McIlvaine's citrate-phosphate buffer pH 7.0) + 0.5 ml. yeast extract (*Haplographium*, *Penicillium* and *Spicaria* 0.125%; *Hormodendrum* 0.48% (w/v)). Centre cup contained 0.2 ml. 5% (w/v) KOH. 0.5 ml. substrates tipped in from side arms—*p*-hydroxybenzaldehyde, syringaldehyde and vanillin 0.1% (w/v); ferulic acid 0.05% (w/v).

Substrate	μl. oxygen uptake over the 5 hr. period following the addition of substrates			
	<i>Haplo-graphium</i>	<i>Hormo-dendrum</i>	<i>Penicillium</i>	<i>Spicaria</i>
None	15.8	31.3	85.8	121.9
<i>p</i> -Hydroxybenzaldehyde	291.7	547.3	364.7	197.7
Ferulic acid	111.1	357.2	114.4	143.1
Syringaldehyde	36.4	225.3	104.7	135.2
Vanillin	332.8	719.0	108.5	156.6

increase with it, while *Hormodendrum* sp. gave an increase which was much lower than was shown with the other phenolic compounds. All four species removed ferulic acid completely during the growth period and all four gave increased oxygen uptakes with this substance. Thus all the results obtained from the Warburg experiments were fully in accord with the previous observations made with these organisms.

Further experiments were carried out in which intermediate products of the metabolism of *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin were added as substrates. Previously it was shown that ferulic acid and vanillin are converted to vanillic acid and syringaldehyde to syringic acid (Henderson & Farmer, 1955). Evidence has now been obtained (Henderson, unpublished) that *p*-hydroxybenzaldehyde is converted to *p*-hydroxybenzoic acid by *Hormodendrum* sp. and *Penicillium* sp. These acid intermediates were added to the Warburg flasks to give a final concentration of 0.0083 M when mixed with the other contents. The most noticeable feature of the respiration rates (Table 2) was the slow rate at which syringic and vanillic acids were oxidized. *Penicillium* sp. and *Spicaria* sp. showed no increase over the endogenous rate on addition of these acids. *Hormodendrum* sp. showed a very small increase with syringic acid but vanillic acid was oxidized at a more rapid rate, while *Haplographium* gave a small increase with both acids. *p*-Hydroxybenzoic acid was oxidized at an increased rate by all four fungi.

The rapid rate of oxygen uptake with *p*-hydroxybenzoic acid compared with the other two acids, may explain the failure to detect it as an intermediate in ordinary growth culture media. It is probable that it is further metabolized whenever it is formed and may therefore never accumulate in the media in any quantity. Neither *Haplographium* sp. nor *Hormodendrum* sp. left any trace of any of the acids in the media during the 21-day growth experiments, but

Table 2. *Respiration of Haplographium sp., Hormodendrum sp., Penicillium sp. and Spicaria sp. on p-hydroxybenzoic, syringic and vanillic acids*

1 ml. of spore suspension added to vessels (*Haplographium* 7.1×10^8 ; *Hormodendrum* 6.1×10^8 ; *Penicillium* 1.1×10^9 ; *Spicaria*? spores/ml.) + 0.5 ml. mineral salts solution + 0.5 ml. buffer (*Haplographium* and *Hormodendrum* Sørensen's phosphate buffer pH 5.3; *Penicillium* McIlvaine's citrate-phosphate buffer pH 3.0; *Spicaria* McIlvaine's citrate-phosphate buffer pH 7.0) + 0.5 ml. yeast extract (*Haplographium*, *Penicillium* and *Spicaria* 0.125 %; *Hormodendrum* 0.48 % (w/v)). Centre cup contained 0.2 ml. 5 % (w/v) KOH. 0.5 ml. substrate (0.05 M) tipped in from side-arms.

Substrate	μ l. oxygen uptake over the 5 hr. period following the addition of substrates			
	<i>Haplographium</i>	<i>Hormodendrum</i>	<i>Penicillium</i>	<i>Spicaria</i>
None	22.1	29.5	176.1	204.5
<i>p</i> -Hydroxybenzoic acid	273.7	68.7	351.9	273.7
Syringic acid	49.5	39.4	167.7	205.8
Vanillic acid	37.4	70.7	168.5	190.4

Penicillium sp. and *Spicaria* sp. left some syringic acid and vanillic acid. The results from the respiration experiments with these species indicate that the acids are more slowly attacked than the corresponding aldehydes, which may explain the accumulation of the acids in the culture media as found with several fungi (Henderson & Farmer, 1955).

Use of spore suspensions for adaptation studies

The curves for rate of oxygen uptake for some of the fungi in the presence of the acid intermediates suggested that they were being oxidized by adaptive enzymes. Therefore the technique for studying the metabolism of fungal spores in the Warburg apparatus was modified according to the exposure technique of Silliker & Rittenberg (1951) to enable investigations into the possible occurrence of adaptation to be made (see Stanier, 1947). For this purpose the spores were incubated overnight in the presence of the substrates, during which time the latter were completely or almost completely utilized. In order to test for any residual respiration a control flask was kept to which only water was added. The presumed adaptive response was shown most clearly in the absence of yeast extract, which was therefore omitted in this part of the work. In the morning the taps were closed and readings were taken at 30 min. intervals for a period of 1.5–2 hr., whereupon the suspected intermediates were tipped in from the side arms. *Haplographium* sp., *Hormodendrum* sp. and *Penicillium* sp. were used for these studies.

Hormodendrum sp. spore suspensions preincubated with *p*-hydroxybenzaldehyde, ferulic acid, vanillin or syringaldehyde gave positive adaptive responses with the corresponding acids known to be intermediates in their

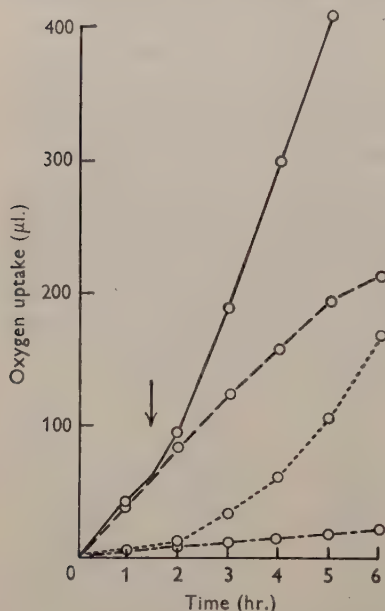


Fig. 1

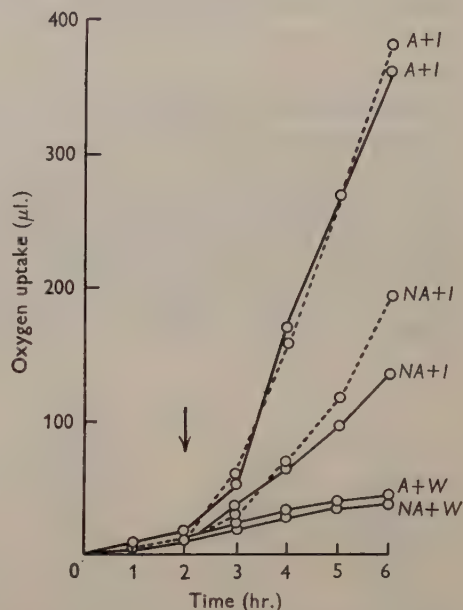


Fig. 2

Fig. 1. *Hormodendrum* sp. Adaptation studies. *p*-Hydroxybenzaldehyde : *p*-hydroxybenzoic acid. Each vessel contained: 1 ml. spore suspension (5.5×10^8 spores per ml.) + 0.5 ml. Sørensen's phosphate buffer pH 5.3 + 0.5 ml. mineral salts solution + 0.5 ml. *p*-hydroxybenzaldehyde (0.1 %, w/v) or 0.5 ml. water. Centre cup contained 0.2 ml. 5 % (w/v) KOH. 0.5 ml. *p*-hydroxybenzoic acid (0.05 M) or 0.5 ml. water tipped in from side-arm. —, Spores adapted by overnight incubation with *p*-hydroxybenzaldehyde; - - - - -, spores incubated overnight without *p*-hydroxybenzaldehyde. *p*-Hydroxybenzoic acid added to these two where indicated by arrow. ---, spores incubated overnight with *p*-hydroxybenzaldehyde, water added where indicated by arrow; - - - - -, spores incubated overnight without *p*-hydroxybenzaldehyde, water added where indicated by arrow.

Fig. 2. *Hormodendrum* sp. Inhibition by citrinin of adaptation to *p*-hydroxybenzoic acid. Spore suspension contained 4.0×10^8 spores per ml. Each vessel contained: 1 ml. spore suspension + 0.5 ml. Sørensen's phosphate buffer pH 5.3 + 0.5 ml. mineral salts solution + 0.5 ml. *p*-hydroxybenzaldehyde (0.1 %, w/v) or 0.5 ml. water. Centre cup contained 0.2 ml. 5 % (w/v) KOH. 0.5 ml. citrinin (500 μ g./ml.) or 0.5 ml. water added to main compartment in morning. 0.5 ml. *p*-hydroxybenzoic acid (0.05 M) or 0.5 ml. water tipped in from side arms. A, spores adapted by overnight incubation with *p*-hydroxybenzaldehyde; NA, spores incubated overnight with water, therefore non-adapted; —, citrinin added at 0 hr.; - - - - -, water added at 0 hr.; I, *p*-hydroxybenzoic acid added where indicated by arrow; W, water added where indicated by arrow.

breakdown (Henderson & Farmer, 1955). Thus it can be seen from Fig. 1 that previous incubation in the presence of *p*-hydroxybenzaldehyde led to an immediate uptake of oxygen on addition of *p*-hydroxybenzoic acid. Where incubation had taken place in the absence of *p*-hydroxybenzaldehyde there

was a period of slow oxygen uptake following the addition of *p*-hydroxybenzoic acid before the maximum rate was reached. Similar results were obtained with the other compounds. During the same period, *Haplographium* sp. and *Penicillium* sp. showed adaptation only to *p*-hydroxybenzoic acid and did not give any increase in the oxygen uptake in the presence of syringic and vanillic acids. However, the growth experiments had revealed that *Penicillium* sp. did decompose vanillic acid, although the syringic acid formed from syringaldehyde accumulated in the medium and was not further decomposed. Therefore it seemed probable that a longer period of incubation and adaptation might lead to oxidation of vanillic acid. In order to start growth the spores were incubated for 16 hr. in the presence of yeast extract. Vanillin was then tipped in and after a further 8 hr. of incubation vanillic acid was added. Following the addition of the acid the spores were incubated for another 16 hr. before the oxygen uptake was recorded. The increase in uptake, compared with the control, showed that vanillic acid was now being oxidized. A similar long experiment in which vanillin and vanillic acid were replaced by syringaldehyde and syringic acid confirmed that *Penicillium* sp. did not oxidize syringic acid, since no additional oxygen was consumed in its presence.

Inhibition of adaptive responses of spore suspensions by antibiotics

Evidence has been obtained which indicates that certain antibiotics function by inhibiting adaptive enzyme formation. Fitzgerald & Bernheim (1948) found that streptomycin inhibited the formation of benzoic acid oxidase, an adaptive enzyme, in mycobacteria. Streptomycin, citrinin and thiolutin were therefore tested for their ability to inhibit adaptive enzyme formation with the organisms used here. The responses were found to vary. Streptomycin at 300 $\mu\text{g./ml.}$ had no inhibitory effect, whereas Fitzgerald & Bernheim (1948) obtained complete inhibition at 200 $\mu\text{g./ml.}$ Citrinin at 83 $\mu\text{g./ml.}$ decreased adaptation by *Hormodendrum* sp. to *p*-hydroxybenzoic, vanillic and syringic acids. The effect was shown most clearly with *p*-hydroxybenzoic acid (Fig. 2). When the acid oxidase had been formed as a result of incubation in the presence of *p*-hydroxybenzaldehyde, the addition of *p*-hydroxybenzoic acid caused an immediate increase in the rate of oxygen uptake, whether citrinin was present or not. However, when there was no period of preincubation with the precursor, citrinin decreased considerably the rate of oxygen uptake as compared with that found for the flask to which no citrinin had been added, and the typical adaptive curve was not produced. Citrinin at 83 $\mu\text{g./ml.}$ did not affect the adaptation of *Haplographium* sp. and *Penicillium* sp. to *p*-hydroxybenzoic acid but at 357 $\mu\text{g./ml.}$ adaptation was almost completely stopped.

The influence of citrinin in decreasing the formation of enzyme required for oxidation of vanillic acid by *Hormodendrum* sp. has been confirmed by a method based on Kluyver & van Zijp's (1951) technique. Mats of the fungus were grown by inoculating with spores 100 ml. volumes of mineral salts medium (see methods), to which were added yeast extract (0.5 %, w/v) and glucose (1 %, w/v). The cultures were grown in 250 ml. conical flasks. After 7 days of incubation at 21° the medium was poured off and the mats washed three times

with sterile distilled water. Citrinin (500 $\mu\text{g./ml.}$) was present in the solution of vanillin (0.1 %, w/v) added to the mats. After 5 days of incubation, analysis of the solution by paper chromatography (Henderson & Farmer, 1955), revealed that there was a considerable amount of vanillic acid present in the culture containing citrinin, and in the control to which no citrinin had been added. After 10 days of incubation there was still a large amount of vanillic acid in the citrinin-containing culture but none remained in the control which indicated that citrinin was preventing decomposition of the acid.

Thiolutin was found to be non-specific for adaptive enzyme formation; it inhibited the oxidation of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid to the same degree.

DISCUSSION

Spore suspensions have been used previously for respiration studies in the Warburg apparatus (Mandels, 1947; Shirk & Byrne, 1951), but these investigations were made directly after harvesting. In the present work, however, the spores were preincubated for a period of 16–17 hr., usually in the presence of yeast extract, which is known to induce rapid germination (Mandels, 1947). The use of spore suspensions as here reported is superior in several ways to the methods which use grown mycelium. One advantage is the uniformity, both quantitative and physiological, which can be obtained between vessels. When using mycelium, discrepancies arise on account of the varying water contents of the samples and the varying physiological activities within the material which consists of hyphae of different ages. A further advantage is that in studying the activity of germinating spores, the physiology of a whole organism is being considered. This is not so when working with mycelial suspensions which contain fragments of mycelium consisting of a few cells. The metabolism of the cells is disorganized in the preparation of the suspensions, as shown, for example, by Darby & Goddard (1950) who found that the Q_{O_2} was influenced by the extent of blending, increasing maceration leading to decreasing activity.

The low rate of endogenous respiration in germinating spores as compared with the very high rates with mycelia is also an important factor. The high endogenous rates with mycelia mask much of the effect on oxygen uptake brought about by the addition of utilizable substrates.

The results presented in this paper confirm the findings previously obtained by growth experiments and analyses of culture fluids (Henderson & Farmer, 1955) concerning the ability of certain fungi to metabolize *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde and vanillin. Both investigations showed that *p*-hydroxybenzaldehyde was more rapidly oxidized than were the other compounds. The presence of one or two methoxyl groups attached to the benzene ring in the other compounds may account for the slower rate at which they were attacked. Support for this view is obtained from the fact that syringaldehyde, with two methoxyl groups, was more slowly attacked than were ferulic acid and vanillin with one each.

In addition, information has been obtained on the process of oxidation of the intermediate products, *p*-hydroxybenzoic, syringic and vanillic acids. A study

of the rate of oxygen uptake in the presence of these compounds showed that they are adaptively attacked. Since preincubation of the spores in the presence of *p*-hydroxybenzaldehyde, ferulic acid, syringaldehyde or vanillin eliminated the lag period before the maximum rate of oxygen uptake was reached after addition of the intermediates, the following conversions appear to be confirmed: *p*-hydroxybenzaldehyde → *p*-hydroxybenzoic acid; ferulic acid and vanillin → vanillic acid; syringaldehyde → syringic acid.

I wish to thank Dr D. M. Webley for his interest and advice and Miss D. Brebner for technical assistance. I am also grateful to Professor A. Robertson for giving me a sample of citrinin.

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A Modified Method for Preparing and Counting Viable Suspensions of Tubercle Bacilli

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SUMMARY: A modified method derived from that of Fenner, Martin & Pierce (1949) is described for the preparation of homogeneous suspensions of single organisms of one strain of *Mycobacterium tuberculosis* at concentrations of about 10^8 single organisms per ml. A technique is also described for the accurate assessment of viable counts in a period of 14 days.

In the study of experimental respiratory tuberculosis a reliable method is required for counting viable organisms of *Mycobacterium tuberculosis* in the suspensions to be sprayed and in collected samples of the cloud the animals inhale. Accuracy of count in the shortest possible time is a first essential. The technique must also be sufficiently simple and safe to allow assessment of at least ten samples with many replicates in the course of any one day of experiment. Further, the technique in use for the production of the clouds of tubercle bacilli (Henderson, 1952; Druett & May, 1952) requires a suspension with an initial concentration of the order of 10^8 organisms/ml. The term 'organism' is here defined as a single rod-like structure which may be composed of one to about five cells of *M. tuberculosis*. When handling suspensions consisting mainly of single organisms this concentration has the added advantage of allowing total counts to be made easily and with reasonable accuracy. In preliminary work we used the technique of Fenner *et al.* (1949) (together with many modifications) and applied it to a variety of strains including one (H37Rv) used by them. Suspensions with a high percentage of single units were obtained, but rarely at a final concentration greater than 10^4 and never higher than 10^6 /ml. Further, no success followed concentration by centrifugation of these suspensions for intractable clumping invariably occurred as a result.

The present paper describes a modification of the technique of Fenner *et al.* (1949) which has been used continuously for over a year in these laboratories and which fulfils most of our requirements. During this time many experiments have been made with airborne clouds of *Mycobacterium tuberculosis*. For these tests a bovine strain was selected, partly because it had high virulence for guinea-pigs, but principally because by suitable methods of cultivation suspensions could be obtained consisting almost wholly of single organisms. This latter property was very desirable for two reasons: first, it ensured that highly homogeneous clouds of single organisms could be produced for inhalation by experimental animals; and second, it allowed the viable count technique to be put to rigorous test.

METHODS

Organism and method of culture. The Vallée strain of *Mycobacterium tuberculosis* var. *bovis* was used. It was stored in small tubes as freeze-dried infected mouse spleen. We have used various methods for recovering and subculturing the strain, but find the following the most satisfactory. Dubos liquid Tween-albumin medium as modified by Fenner *et al.* (1949) was the principal culture fluid; the amino acid constituents were added in the form of 0.05 % (w/v) Bacto Casamino Acids (Difco), and the medium adjusted to pH 7.0. The content of one tube of freeze-dried material was taken up in this medium and then inoculated on to Dorset egg medium and incubated at 37° for 7 days. Subculture was then made on Dubos liquid medium and thereafter subculture was made on the same substrate at 7-day intervals. The medium was contained in 8 × 5 in. Roux bottles, each containing 100 ml. and a 5 % (v/v) inoculum was used. The resulting depth of liquid seemed optimal for maximum yield of viable organisms. Three to four subcultures in this medium were necessary before maximum growth of the right consistency was obtained for experimental cloud work. Thereafter, five to eight subcultures could be made without loss of virulence before reverting to another tube of freeze-dried material.

Preparations of suspensions. Immediately on removal from the incubator and before cooling to room temperature the cultures were passed through sterile folded filter-paper (Green's hydruo no. 904½). 80 % of the particles in the filtrate were single units of 1 μ .³ or less. The remainder were small clumps containing two or three units each in a total viable population of $1-2 \times 10^8$ organisms/ml.

Presumptive viable count. Total counts on the suspension were immediately made in a Petroff-Hausser bacterial counting chamber, with dark-ground illumination. By comparing these total counts with those obtained by culture, accumulated evidence showed that the viable count was consistently of the order of 80 % of the total count. This presumptive test finally formed a basis which allowed experiments to be made immediately with the freshly prepared suspensions.

Microvessels for colonial culture. Small lidless dishes of 35 mm. diameter, 10 mm. depth, with walls 2 mm. thick, and having optically flat bases, were made for us by Messrs R. and J. Beck, Ltd., London. Each of these microvessels was contained in a 2 in. diameter Petri dish. The latter were placed in 7 lb. Kilner jars complete with glass lids and screw-retaining rings but with no rubber seal. In this manner the microvessels were hot-air sterilized and stored ready for use.

Plating medium. Dubos agar medium, as modified by Fenner *et al.* (1949), was prepared monthly in 100 ml. screw cap bottles. As with the liquid medium, the amino acid content was added as 0.05 % (w/v) Bacto Casamino Acids (Difco); the agar used was British Drug Houses 'Japanese fine powder'. The pH value was adjusted to pH 6.5 and great care was taken in filtration to ensure an optically clear gel. Immediately before use 5 ml. of 10 % (w/v) bovine

plasma albumin (Armour Laboratories, Fraction V) in saline (pH 6.5) and 1 ml. of 50 % (w/v) citrated glucose were added to each 100 ml. of the melted agar at 56°. No Tween or oleic acid was incorporated in the medium because these substances markedly inhibited colony development and those colonies which appeared varied widely in size. Each microvessel was filled with 5 ml. of medium. After the agar had jelled the microvessels were incubated at 37° in the inverted position in their containing Petri dishes for 18–24 hr. When not required immediately they were stored in Kilner jars at 0–4°. Before use the dishes, removed from the Kilner jar, were warmed in an incubator at 37° for 1 hr.

Inoculation of microvessels. Dilutions of the bacterial suspension were made in 0.1 % (v/v) bovine plasma albumin in such a manner that 0.01 ml. of the appropriate dilution was calculated from the presumptive viable count to contain 30–150 colonies. Three 0.01 ml. volumes were delivered on to the dried agar surface of each microvessel from a sterile 0.1 ml. blood pipette controlled by a special metering device (see Appendix) which allowed high accuracy of delivery.

Incubation of the microvessels. After inoculation the microvessels were left unmoved until the deposited liquid had spread over and sunk into the agar. This occurred within a few minutes when the organisms were inoculated while still warm. The microvessels, each contained in a 2 in. Petri dish, were then incubated in the inverted position in 7 lb. Kilner jars each carrying four columns of Petri dishes, totalling 32–34/jar. A test-tube with water was placed in a centre space formed by the dishes and served to prevent desiccation of the medium. The glass lids were replaced on the Kilner jars and secured by the metal screwband only. Incubation was carried out at 37°. Colonies just visible to the naked eye were seen after 14 days' incubation. On removal from the incubator there was no evidence of desiccation of the agar surface or condensation of water within the jars. While the dishes were still warm, a drop (*c.* 0.05 ml.) of 40 % (w/v) formaldehyde was placed on the underside of the lids of the Petri dishes, which were then replaced in the jar as for incubation. It was found that after 4 hr. at 37° the microvessels were sterile. Colonies removed by platinum loop and transferred to 10 ml. amounts of Dubos liquid medium consistently failed to grow even after prolonged incubation at 37°; untreated colonies similarly tested consistently yielded fully grown cultures within 10 days of incubation.

Colonial counts aided by optical projection. Repeated tests showed that there was no increase in the number of colonies after 14 days. However, their size at this time prevented accurate counting by the naked eye. Counting could be done by examination under a plate microscope, but when large numbers of microvessels were in use this process quickly led to fatigue and inaccuracy. Counting was made much easier by the use of an optical projection system designed to accommodate the microvessels. The projector was arranged to throw an image of the inoculated area on a glass bead screen in a darkened room. The optically flat bases of the microvessels prevented image distortion, and large numbers of counts could thus be quickly and accurately made.

The projector lens was a cinematograph anastigmat of focal length 2 in. and aperture *f.* 1.6, giving a wide flat field. The remainder of the apparatus, consisting of a force-cooled projection lamp and condenser, was of conventional design. The magnification of the image was about $\times 80$ at 14 ft. from the projector. For convenience in counting, the glass bead screen was divided by black cotton thread into 4 in. squares. By this method all colonies grown from one measured drop were illuminated in one field. The optimum number of colonies for counting was probably about 50, but reasonable accuracy could be expected in the range of 30–150 colonies. Plate 1 shows the type of picture encountered. Each colony developed a well-marked central spot. This spot was a particularly useful aid when several colonies arose close together, as seen in the upper left-hand part of Fig. 1. The extreme bottom right-hand corner of Pl. 1, fig. 1 shows, in partial illumination, colonies forming part of another measured drop. Also in the right-hand corner there is seen a well-defined 'dust' spot. This minor defect was not uncommon, but there was no difficulty in deciding which was, or was not, a colony of tubercle bacilli.

RESULTS

In eighteen consecutive experiments with clouds of single organisms, the suspension used was tested for viable count before and after the experiment, and two to ten collected cloud samples per experiment were similarly tested. The number of replicate microvessels used in any assay of suspension or cloud sample varied from three to four but there was a 2–3 % loss as a result of either extraneous contamination or the merging of drops on the agar surface in microvessels.

Viable counts of micro-organisms in equal drops taken from the same suspension may be expected to follow a Poisson distribution, but the experimental errors are such that so far as we are aware the ideal has never been achieved. In fact, it would seem from any published data, or experience in our laboratories, that 90 % of samples giving adequate 'goodness of fit' (Fisher, 1946) represents a high measure of success. The data from the present experiments were examined statistically by our colleague Mr S. Peto. He fitted a Poisson distribution to each assay and examined individual drop counts for aberrant values. Among ninety-six assays there were nine such counts so small or so large as to have a probability of about 1 % (= approximately 1/96) or less, of occurring in the sample to which each belonged. These nine were rejected and new Poisson distributions were fitted to the assays affected. The value of χ^2 was computed for every distribution. The sum of χ^2 for assays of liquid suspensions gave a probability of 10 % ($\Sigma\chi^2 = 358.33$; $n = 36$); for assays of cloud samples the probability was 24 % ($\Sigma\chi^2 = 516.66$; $n = 60$). Finally, there was only one assay (of the first cloud sample taken) that was statistically hopelessly erratic. We consider, therefore, that the soundness of the method is established beyond reasonable doubt.

DISCUSSION

Fenner *et al.* (1949) raised the status of the quantitative assessment of tubercle bacilli from the position of expressing dosage in terms of fractions of a milligram or portions of a loopful to one of reasonable accuracy in terms of viable organisms. The present work is best regarded as introducing modifications to their technique, leading to possible general improvements. For our preliminary studies on airborne infection it certainly has marked advantages. The density of suspensions of single organisms that can be obtained is sufficient to make direct total counts and to produce suitable cloud concentrations to which animals can be exposed. The assay is completed in 14 days. The serious problems of either desiccation of agar or excessive condensation with concomitant contamination during incubation have been simply overcome. Economy in space and materials (particularly in plasma albumin) is, from our point of view, very important. A high degree of precision in counting has been achieved and large numbers of samples can be readily handled at short notice. However, many other strains of *Mycobacterium tuberculosis* would require to be successfully tested before these advantages could be accepted as being generally applicable in studies with this organism.

We are grateful to Dr D. W. Henderson for initiating this research. He has also given valuable assistance in the preparation of the paper. We wish to thank Dr C. Pierce of the Rockefeller Institute for guidance during the time she worked in our Department. We are grateful also to our colleagues Mr E. O. Powell, who designed the optical projection system, Mr Clement, who constructed this instrument and the pipetting device, and Mr S. Peto for the statistical analysis of results of viable counts.

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APPENDIX

Metering device, construction and use

Fig. 1 shows a sketch of the instrument. It consists of a brass cylinder $2\frac{3}{4} \times \frac{3}{4}$ in., bored for a distance of $1\frac{1}{16}$ in. with a $\frac{1}{8}$ in. \times 40 t.p.i. drill and then for a further $1\frac{1}{16}$ in. with a $\frac{3}{16}$ in. drill which was unthreaded. At the other end the cylinder was bored with a $\frac{5}{8}$ in. drill for a distance of $\frac{5}{8}$ in.; this space was filled with a rubber bush for holding a pipette. A metal washer with an internal aperture smaller than the bush was first inserted in the space as a check on the



J. P. ALBERICCI AND J. A. FLETCHER—COUNTING TUBERCLE BACILLI. PLATE 1

(Facing p. 697)

depth to which the pipette could be inserted. A male counterpart for the cylinder was fitted as shown in the figure threaded at one end and made a perfect screw fit by lapping into the cylinder thread. The unthreaded extension of the spindle was made to give a sliding fit with the corresponding part of the cylinder. A 1 in. metal knob was fixed in the spindle to allow of easier rotation by digital pressure. The instrument was finished in dull nickel plate. The threaded parts and plunger were greased to give smooth action and to make an airtight seal.

In operation a sterile pipette with cotton-wool plug is fitted in the rubber bush and the spindle screwed down almost to fullest extent. The pipette is charged by immersing the tip in the bacterial suspension and turning the knob of the spindle anti-clockwise by thumb pressure, until the pipette contains a volume just in excess of 0.1 ml. Then, by clockwise rotation of the spindle, the excess liquid is deposited on to the surface of a spare agar plate. Three microvessels are then inoculated with three volumes of 0.01 ml., the remaining 0.01 ml. being discarded. Each 0.01 ml. is first extruded from the tip of the pipette, which is then placed on the agar and the liquid thus removed. By this means not only accurate volume delivery is secured but splashing is avoided.

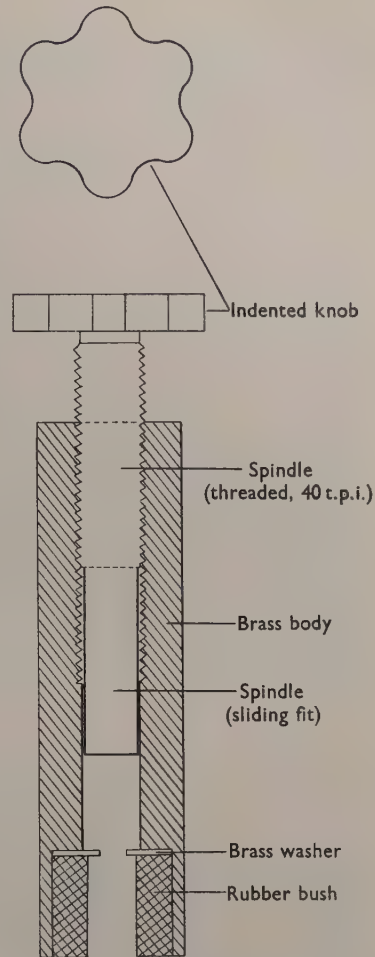


Fig. 1. L.S. pipette metering device ($\times 2$).

EXPLANATION OF PLATE

Fig. 1. Reduction by 6.66 diameters of actual size of projected image.

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Some Mutational Changes in the Photosynthetic Pigment System of *Rhodopseudomonas spheroides*

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SUMMARY: Some 50 mutants, which differ from the wild type in the nature of their pigment systems, were isolated from the non-sulphur purple bacterium, *Rhodopseudomonas spheroides*. They fall into five main groups. The group of colourless mutants is incapable of photosynthetic growth, and devoid both of chlorophyll and carotenoids. The remaining groups still contain bacteriochlorophyll, but differ chemically from the wild type and from one another in their carotenoid pigments; all are capable of photosynthetic growth. The wild type contains two principal carotenoids, one red and one yellow. The dark red mutants contain both wild-type carotenoids and traces of a new red carotenoid. The brown mutants contain approximately equal amounts of the wild-type yellow carotenoid and of neurosporene, together with traces of the wild-type red carotenoid. The green mutants contain neurosporene and a dihydroxy derivative, but neither of the wild-type pigments. The blue-green mutants contain phytoene, a colourless polyene, but are completely devoid of coloured carotenoids. The infra-red spectrum of cells of the blue-green mutant is markedly different from that of the wild type and of the other photosynthetic mutants, despite the fact that its chlorophyll is chemically identical with theirs.

In his classical account of the Athiorhodaceae, van Niel (1944) devoted only one paragraph to the question of variations that affect photosynthetic ability. After describing the gradual weakening of photosynthetic power that occurs when aerobic representatives of the group are carried through many transfers in the dark, he concluded with the statement: 'So far, I have not observed a permanent loss of photosynthetic activity in cultures which have been grown in the dark for many years. Whether by careful selection subcultures might be isolated which have become non-photosynthetic remains a problem for the future.' Subsequent papers on the Athiorhodaceae contain no further information about this aspect of their biology. During the past ten years, however, many mutants with pigment systems differing from that of the wild type have been obtained in other photosynthetic organisms. Mutants of this kind in the green alga *Chlorella* have provided the material for studies by Granick and his collaborators on the biosynthesis of chlorophyll (summarized in Granick, 1954), and by Claes (1954) on carotenogenesis.

During work on the physiology of the non-sulphur purple bacterium *Rhodopseudomonas spheroides*, a spontaneous mutant differing from the wild type in its carotenoid pigments was discovered by Dr Germaine Cohen-Bazire. This observation prompted a more systematic study of mutational changes affecting the pigment system of *R. spheroides*. The results are reported in the present paper.

METHODS

Biological material and media. The wild type employed was *Rhodospseudomonas spheroides*, strain 2.4.1, obtained from the collection of Professor C. B. van Niel of the Hopkins Marine Station of Stanford University. For the isolation of mutants, and for general purposes of cultivation under photosynthetic and non-photosynthetic conditions, the following medium (medium A) was used: ammonium malate 0.8 g., magnesium sulphate 0.05 g., calcium chloride 0.005 g., yeast extract 1.0 g., and 100 ml. 0.05 M-potassium phosphate buffer (pH 6.8). For solid media, 1.0–1.5 % (w/v) of agar was added. Cultures were maintained either photosynthetically as stabs incubated in the light, or aerobically as slopes incubated in the dark. For certain growth experiments, the partially defined medium described by Cohen-Bazire, Sistrom & Stanier (1956) was used (medium B).

Isolation of radiation-induced mutants

Rhodospseudomonas spheroides was grown in medium A under semi-anaerobic conditions (completely filled Florence flasks) in the light. When the culture attained a population density of $2.0\text{--}5.0 \times 10^9$ organisms/ml. a sample was diluted 100-fold, and 5 ml. of this diluted suspension were subjected to ultra-violet irradiation for 90 sec. from a General Electric Germicidal 8 W. lamp, held 14 in. above the Petri dish containing the suspension. Previous experiments showed that this treatment killed 99.9 % of the organisms. The irradiated suspension was diluted to contain about 2000 viable organisms/ml., and 0.1 ml. of this dilution was spread over the surface of each of 50 plates of medium A. After 4–7 days of incubation under aerobic conditions in the dark, the plates were examined for pigment mutants.

Spectrophotometric measurements

A Beckman model DU spectrophotometer was used. The spectra of whole organisms were measured in a special cuvette holder with a pane of opal glass between the cuvettes and the photocell, which reduced the effects of scattering (Shibata, Benson & Calvin, 1954). In order to facilitate comparison, the spectra of whole organisms were measured on suspensions adjusted to a fixed optical density at 680 m μ ., since at this wavelength light absorption by the pigments is negligible.

Isolation and determination of pigments

Bacteriochlorophyll and bacteriopheophytin. Bacteriochlorophyll was measured quantitatively as described by Cohen-Bazire *et al.* (1956). Bacteriopheophytin was prepared by a modification of the method of van Niel & Arnold (1938). The chlorophyll was extracted from 2–5 g. (wet weight) of fresh organisms by two treatments with methanol, a total volume of 20 ml. being used. Ethyl ether (20 ml.) was added, and the bacteriochlorophyll was then converted to bacteriopheophytin by the addition of 2 ml. 5 N-H₂SO₄. Twenty ml. of water were added rapidly before the bacteriopheophytin precipitated out of the acid methanol ether mixture. Bacteriopheophytin and carotenoids

went into the ether phase, which was washed to neutrality, dried, and evaporated to dryness. The residue was taken up in light petroleum (60–70°). To eliminate the carotenoids, the light petroleum extract was chromatographed on a column packed with a mixture of equal weights of MgO and Super Cel; the column was then developed with an acetone+light petroleum mixture. Bacteriophaeophytin ran through the column rapidly and was collected in the effluent. In some cases, passage through a second column was required to eliminate a fast-moving yellow carotenoid.

Carotenoids. Coloured carotenoids were extracted from the finely ground, vacuum-dried organisms with carbon disulphide. This solvent extracts the carotenoids completely, but removes only a small amount of the chlorophyll. The extract was evaporated to dryness *in vacuo* and taken up in light petroleum. The pigments were separated on a magnesium oxide Super Cel (1:1) column, which was developed with 5% (v/v) acetone in light petroleum. Where the separation was inadequate, purification was effected on a calcium hydroxide column, developed with 2.5% (v/v) acetone in light petroleum. The spectra were determined in light petroleum.

Phytoene. Fresh organisms were extracted twice with a mixture of 7 vol. acetone + 1 vol. methanol. The pigments were transferred to a purified light petroleum by the addition of an equal volume of water. The commercial light petroleum used contained substances with a strong ultraviolet light absorption, which interfered with the spectrophotometry of phytoene. Special light petroleum, freed of these substances by passage through a silica gel column, was used in all work with phytoene. Acetone and methanol were removed by washing the light petroleum layer four times with a saturated aqueous solution of sodium chloride and once with water. The use of sodium chloride in the initial washes diminished the formation of emulsions. The extract was dried, concentrated to a small volume, and placed on a short MgO: Super Cel (1:1) column. The chromatogram was developed with light petroleum. Chlorophyll and its degradation products were held at the top of the column, while the phytoene ran through rapidly and was collected in the first 10–15 ml. of effluent. The spectrum was measured in light petroleum. Quantitative estimations of phytoene were based on the absorption at 286 m μ ., using the value of 850 reported by Rabourn, Quackenbush & Porter (1954) for the extinction coefficient ($E_{1\text{ cm.}}^{\%}$).

Preparation and fractionation of bacterial extracts

Organisms were harvested, washed in 0.02 M-potassium phosphate buffer (pH 7.5), and resuspended in the same buffer. The suspension was subjected to sonic oscillation in a Raytheon 9-Kc Magnetostriiction Oscillator for 10 min. to break the organisms and release the chromatophores. Whole organisms and debris were removed by low-speed centrifugation (10,000 g) for 10 min.; the resulting clarified extract was then further separated into a chromatophore fraction and a supernatant fraction by centrifugation for 1 hr. at 144,000 g. For the determination of the spectra of chromatophores, this fraction was resuspended in 0.05 M-potassium phosphate buffer (pH 7.5).

Protein determinations. A sample of bacterial suspension which contained about 1.0 mg. protein was used. The organisms were sedimented, resuspended in 0.1 ml. water and extracted with 4.9 ml. of a 7 : 2 acetone : methanol mixture to remove the pigments which interfered with the colorimetric protein determination. After centrifugation, the extract was discarded and the protein content of the pellet determined by the biuret method (Weichselbaum, 1946).

RESULTS

Pigments of the wild type

Three principal pigments are associated with the photosynthetic apparatus of *Rhodopseudomonas spheroides*: bacteriochlorophyll, the form of chlorophyll found in all purple bacteria (van Niel, 1944); two carotenoids, one yellow and one red (van Niel, 1947). The colour of the organisms varies from brown to bright red, depending on the ratio between the two carotenoids, which changes

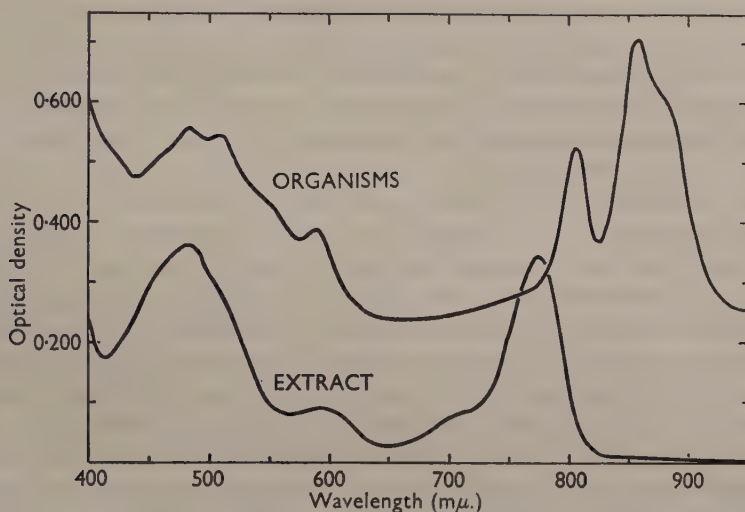


Fig. 1. A comparison of the absorption spectra *in vivo* and *in vitro* of the pigments in the wild type of *Rhodopseudomonas spheroides*. The curve marked ORGANISMS was measured on an aqueous suspension of whole organisms, with opal glass to minimize the effects of light scattering. The curve marked EXTRACT was measured on a methanol+acetone extract which contained all the bacteriochlorophyll and carotenoids, and was adjusted to the same pigment concentration as the cell suspension.

with the conditions of cultivation. Microscopic examination fails to reveal any intracellular localization of the photosynthetic pigments; but in extracts of the organisms, the pigments are found exclusively associated with a sedimentable fraction consisting of particles about 50 mμ. in diameter. These particles, which have been termed 'grana' (Thomas, 1952) and 'chromatophores' (Pardee, Schachman & Stanier, 1952), are presumably the functional equivalents of chloroplasts, although much smaller and apparently of simpler structure.

Fig. 1 shows the absorption spectrum of a suspension of living organisms,

and of the mixed pigments extracted from these organisms by a mixture of methanol and acetone. In the organisms, light absorption by bacteriochlorophyll occurs principally in the infra-red region between 800 and 950 $m\mu$., with peaks at 805 and 855 $m\mu$. and a shoulder at about 875 $m\mu$. There is a minor chlorophyll peak at 590 $m\mu$, and a rising absorption at the short end of the visible spectrum which culminates in the Soret peak at 375 $m\mu$. The region of carotenoid absorption in the organisms lies between 425 and 575 $m\mu$., with peaks about 452, 477 and 508 $m\mu$. It can be seen from Fig. 1 that the positions of the absorption maxima in the spectrum of the extracted pigments are markedly different from the positions of the maxima in the spectrum of the organisms. The spectrum of bacteriochlorophyll shows the greatest displacements: in organic solvents, it has virtually no absorption beyond 800 $m\mu$., the main peak being situated at about 775 $m\mu$., some 80 $m\mu$. short of its position as seen in the organisms. Such major spectral shifts following extraction occur with the pigments of all purple bacteria. In describing the phenotype of *Rhodopseudomonas spheroides* with respect to the pigment system, both the determination of the spectrum of the whole organisms and the characterization of the isolated pigments have proved useful; certain of the changes which occur in mutants can be detected by one method, but not by the other.

The pigment system of *Rhodopseudomonas spheroides* is profoundly affected by the conditions under which the organisms are grown (Cohen-Bazire *et al.* 1956). Maximal concentrations of the photosynthetic pigments occur in organisms cultivated anaerobically in the light. Under such conditions, the pigment concentration is inversely related to light intensity. Furthermore, light intensity affects the shape of the infra-red spectrum: in organisms grown at low light intensities the absorption at 855 $m\mu$. is far higher than at 875 $m\mu$.; but as the light intensity during cultivation is increased, the shoulder at 875 $m\mu$. becomes more and more prominent, eventually emerging as a separate peak at very high light intensities. Oxygen almost completely inhibits synthesis of the photosynthetic pigments, organisms grown in strict aerobiosis being practically colourless. Despite this, colonies on aerobic plates eventually show considerable pigmentation in the central regions, a phenomenon no doubt ascribable to the fact that in a well-developed colony many of the organisms are effectively shielded from contact with oxygen by their neighbours. In addition to suppressing pigment synthesis, oxygen has a special effect on the carotenoids of *R. spheroides*. The proportion of the red carotenoid is always much higher in organisms exposed to air than in organisms grown under strict anaerobiosis. Hence colonies or flask cultures exposed to air are red, whereas stabs or bottle cultures are brown. When an anaerobically grown culture is exposed to air, it turns red in a short period of time. A quantitative study of the pigment changes that occur (van Niel, 1947) strongly suggests that there is a stoichiometric conversion of the yellow carotenoid to the red one, although this conclusion has been questioned recently (Goodwin, Land & Osman, 1955).

It is clear from this discussion that an accurate characterization of the phenotype with respect to pigmentation is possible only when the organisms are grown under closely controlled and reproducible conditions. Unless otherwise

specified, all reported data on pigments have been obtained with organisms grown anaerobically (atmosphere: 95 % N_2 + 5 % CO_2) in medium A at a temperature of 30° and at a uniform light intensity close to that saturating for growth, and harvested during the exponential phase of growth. These conditions cannot be used, of course, for the cultivation of non-photosynthetic mutants, which were grown at 30° semi-aerobically in the dark in medium A.

Spontaneous variation in the wild type

Cultures of the wild type maintained anaerobically in the light (i.e. under conditions where photosynthesis is obligatory) show little tendency to produce spontaneous pigment mutants. On two occasions, however, spontaneous mutants of the *green* phenotype, which is described in a later section, were isolated from photosynthetic cultures of the wild type. Since these mutants have the same photosynthetic growth rate as the wild type, they are presumably not eliminated by selection in anaerobiosis and light.

Grown aerobically in the dark, the wild type is far less stable. After repeated transfers under these conditions, two mutants make their appearance. One produces colonies which are much paler than those of the wild type, while the other produces colourless colonies. The former can still grow photosynthetically: the latter cannot, but will revert to the wild type again when placed under photosynthetic growth conditions. A similar pattern of genetic instability during aerobic growth in the dark is shared by the various classes of photosynthetic mutants to be described below. We have not made a detailed study of these spontaneous variants. Presumably during aerobic growth, mutants with a lessened ability to manufacture the photosynthetic pigments are not eliminated (and perhaps even favoured) by selection, thus gradually accumulating in the population. In so far as they have been examined, the pale phenotypes do not differ qualitatively from their parental stocks with respect to their pigments. This type of variation no doubt explains the physiological observations made by van Niel (1944):

‘Aerobic representatives may, of course, be kept on ordinary slants and grown in the dark. However, it is my impression that under such conditions the photosynthetic ability of the organisms slowly weakens. I have had cultures of a number of strains both in stabs, exposed to continuous illumination, and on slants which were regularly kept in the dark except at times when they were transferred. In the course of ten to fifteen years the ‘dark cultures’, though still capable of slow and scanty development in the light, were decidedly less suitable for photosynthesis experiments than the corresponding stab cultures. By a process of selection one can succeed in gradually restoring the original vigour, but this takes time, and many transfers in media where growth depends on photosynthetic activity are required to achieve it.’

The isolation of induced mutants

On aerobic plates spread with irradiated suspensions and incubated in the dark at 30°, the central portions of the colonies have become sufficiently pigmented after 4–7 days to permit visual discrimination between colonies of

the mutants and of the wild type. In all, about 50 mutants were isolated. On the basis of their colour, they were divided into five main phenotypes: *dark red*, *brown*, *green*, *blue-green* and *colourless*. Mutants of the green phenotype were most common, followed in order by dark red, brown, blue-green and colourless. All save those of the colourless phenotype proved to be still capable of photosynthetic growth, and a more refined specification of the phenotypes could then be made by comparing the absorption spectra of the organisms, grown anaerobically in the light, with the spectrum of the wild type organisms grown under the same standard conditions; Fig. 2 shows some representative spectra. That of the dark red phenotype is not included, since the spectrum of these mutants does not differ detectably from that of the wild type.

In organisms of the brown and green phenotypes, the chlorophyll peaks (375, 590, 805, 855 m μ .) are identical with those of the wild type. In the region of carotenoid absorption, the brown phenotype shows peaks at 430, 459 and 491 m μ ., all displaced to considerably shorter wavelengths than those of the wild type. The peaks in the carotenoid region of the spectrum of the green phenotype lie close to those of the brown, at 430, 457 and 488 m μ .; but the relative heights are very different, and there is also a marked decrease in absorption around 550 m μ . The spectrum of the blue-green phenotype differs from that of the wild type in the regions of both chlorophyll and carotenoid absorption. Chlorophyll peaks at 375 and 590 m μ . are still present, but in the infra-red region the peak of the wild type at 805 m μ . has been replaced by a long shoulder around 810 m μ ., and the major peak of the wild type at 855 m μ . has disappeared, giving place to a completely symmetrical peak with a maximum at 877 m μ . There are no peaks whatsoever in the carotenoid region, and the general lowering of the absorption suggests the total absence of carotenoid pigments. In shape (though not in the absolute positions of the peaks), the spectrum of the blue-green phenotype bears a considerable resemblance to that of pure bacteriochlorophyll in organic solvents.

Lastly, the spectrum of a representative of the colourless phenotype (grown semi-aerobically in the dark) is shown in Fig. 2. There are no characteristic peaks in the regions either of chlorophyll or of carotenoid absorption; the small isolated peak at 420 m μ . probably reflects cytochrome absorption.

In order to determine the intracellular localization of the pigments in the various photosynthetic mutants, cell extracts were made, the chromatophore fractions were isolated by differential centrifugation, and spectra were measured on these fractions. The chromatophore suspensions had spectra virtually identical, both in general shape and in the position of the maxima, with those of the organisms from which they were prepared. It thus appears that the changes in the pigment systems of the mutants have occurred within the chromatophores.

Identification of pigments in the photosynthetic mutant phenotypes

A single strain of each photosynthetic phenotype was selected for chemical study of the pigments and comparison with those of the wild type.

Chlorophyll. Since bacteriochlorophyll is somewhat unstable, it was identi-

fied by the preparation of the phaeophytin derivative, as described in Methods. The spectra in chloroform of the phaeophytins prepared from the wild type, and from the dark red, brown, green and blue-green mutants were identical. Furthermore, they coincided with the spectrum of purified bacteriopheophytin

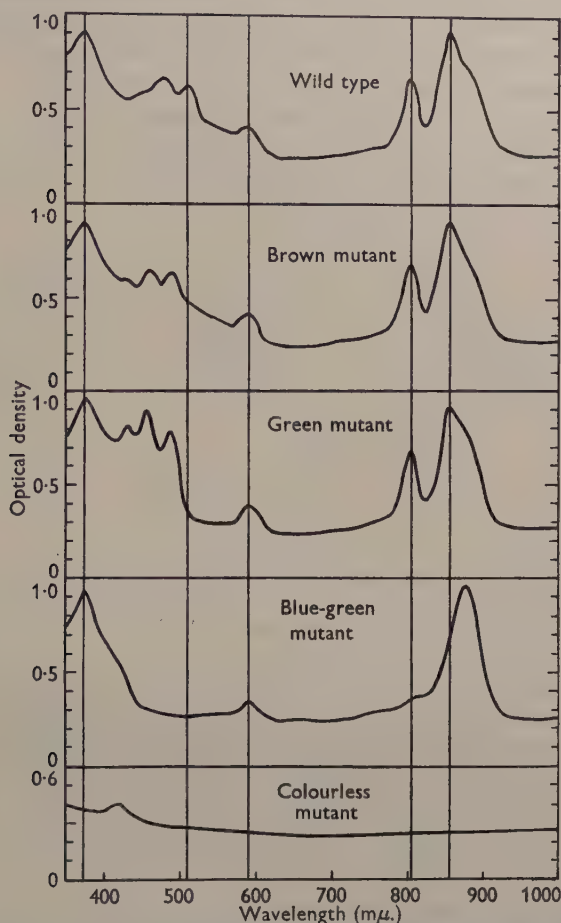


Fig. 2. Absorption spectra of whole organisms of the various phenotypes of *Rhodospseudomonas spheroides*. All spectra were adjusted to the same optical density at 680 $m\mu$. Vertical lines have been drawn through some of the maxima in the spectrum of the wild type to facilitate comparison.

from *Rhodospirillum rubrum*. Since the only change that occurs in the conversion of a chlorophyll to a phaeophytin is the elimination of magnesium from the molecule, it seems evident that all the strains examined contained bacteriochlorophyll. This might have been anticipated for the dark red, brown and green mutants, whose cells show the same chlorophyll absorption as does the wild type. However, as mentioned above, the infra-red spectrum of the blue-green mutants differs markedly from that of the wild type. The identity of the chlorophyll in this mutant was therefore tested more directly. The

organisms were extracted with methanol, and the spectrum of the methanolic extract was compared with that of pure bacteriochlorophyll in the same solvent. Since the blue-green mutant contains no coloured carotenoids, a chromatographic purification of the methanolic extract was unnecessary for spectral comparison. The extract showed complete spectral identity with bacteriochlorophyll over the range from 350 to 800 $m\mu$. It may therefore be concluded that the anomalous infra-red spectrum of the blue-green mutant is not caused by a change in the chemical nature of its chlorophyll.

Coloured carotenoids. The carotenoids in mutants of the green, brown and dark red phenotypes were isolated as described in Methods and compared with those of the wild type. All the spectra (Fig. 3) were measured in light petroleum,

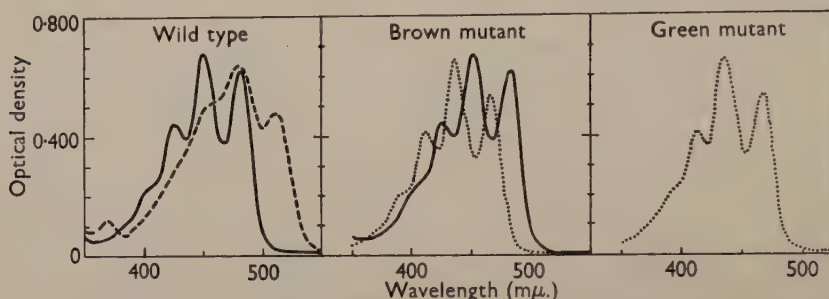


Fig. 3. Absorption spectra, measured in light petroleum, of the principal carotenoid pigments present in the wild-type and in the green and brown mutants of *Rhodospseudomonas spheroides*. Solid line=yellow pigment of wild type. Dashed line: red pigment of wild type. Dotted line=yellow pigments of mutant (neurosporene and dihydroxyneurosporene).

and the reported absorption maxima refer to this solvent. The yellow carotenoid of the wild type had three very sharp peaks at 426, 451 and 481 $m\mu$; the red carotenoid of the wild type had relatively broad peaks at 481 and 512 $m\mu$, together with a shoulder at 451 $m\mu$.

The green mutant contained neither pigment of the wild type, but instead two new yellow carotenoids, present in approximately equal amounts. These pigments have identical spectra, similar in shape to that of the yellow pigment from the wild type, but with peaks at 413, 437 and 467 $m\mu$. They are separable from one another owing to their markedly different rates of movement on columns. Nakayama & Chichester (unpublished studies) have crystallized both these substances, and find that their specific extinction coefficients are slightly different. The one that moves most rapidly appears to be identical with neurosporene, a carotenoid found in *Neurospora* sp. by Haxo (1949); the other is a dihydroxy derivative of neurosporene. Nakayama & Chichester have also found very small traces of neurosporene in extracts of the wild type organisms.

The brown mutant contains the yellow carotenoid of the wild type and the more rapidly moving of the two carotenoids found in the green mutant (neurosporene). In addition, very small quantities of the red carotenoid of the wild type are present.

The dark red mutant contains both the carotenoids of the wild type, together with traces of a second red carotenoid, which moves very slowly on columns, even when a 1 : 1 mixture of acetone + light petroleum is used for development. This pigment has a spectrum similar in shape to that of the wild-type red carotenoid, but with maxima at 462, 490 and 522 $m\mu$. It has not been obtained in sufficient quantities for further characterization.

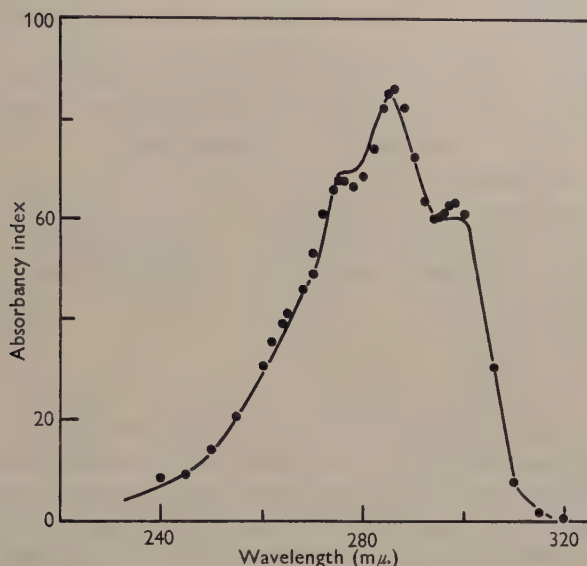


Fig. 4. Absorption spectrum of crude phytoene extracted from the blue-green mutant of *Rhodospseudomonas spheroides*, compared with that of purified phytoene from tomato fruit. Both curves adjusted to the same optical density at 285 $m\mu$. Solid line = purified phytoene from tomato (data of Dr J. W. Porter). Points = phytoene of the blue-green mutant of *R. spheroides*. According to Dr Porter (personal communication), the secondary maxima at 275 and 298 $m\mu$ in crude phytoene from tomato are considerably more pronounced than in the purified material. Hence the differences in this respect shown in the graph probably reflect the fact that the bacterial phytoene has not been purified.

Colourless polyenes. Chromatographic analysis of an extract of the pigments from the blue-green mutant confirmed the absence of coloured carotenoids, already strongly suggested by the absorption spectrum of the organisms. Apart from bacteriochlorophyll and its degradation products, the only coloured band on the column was a faint yellow one with a single symmetrical peak at 280 $m\mu$. and a slowly decreasing absorption in the visible region between 400 and 500 $m\mu$. This substance has not been identified, but from its spectrum it is evidently not a carotenoid. When effluents from columns treated with extracts of the blue-green mutant were examined spectrophotometrically, large amounts of a colourless non-fluorescent substance with a strong absorption in the ultra-violet were found; all of this material was contained in the first 10–15 ml. of light petroleum which emerged from the column. The behaviour of this material on the column and its absorption spectrum (Fig. 4) suggest that it is phytoene,

a C₄₀ polyene originally found by Porter & Zscheile (1946) in tomato fruits. The related colourless polyene, phytofluene (Wallace & Porter, 1952) does not appear to occur in the blue-green mutant; it is a fluorescent substance, and the chromatograms of extracts from the blue-green mutant do not show any colourless fluorescent bands.

Since an absorbancy index for phytoene has been published (Rabourn *et al.* 1954), it was possible to determine roughly the amount of phytoene present in the blue-green mutant, by measuring the total ultraviolet absorption of the effluents from columns treated with extracts prepared from a known quantity of cell material. Such determinations show the presence of 1–3 mg. phytoene/g. bacterial protein in photosynthetically-grown organisms. This is of the same order of magnitude on a weight basis as the total quantity of coloured carotenoids in organisms of the wild type grown under similar conditions. Examination of the appropriate fraction from columns treated with extracts of the pigments from wild type organisms failed to reveal any phytoene (limits of detection; about 1 % of the concentration present in the blue-green mutant). Goodwin *et al.* (1955) reported the absence of phytoene from organisms of the wild type.

Localization of phytoene in the blue-green mutant

Phytoene cannot be directly detected by the spectrophotometric examination of whole organisms or chromatophores, since its absorption spectrum is masked by scattering and by the ultraviolet absorption of the nucleic acids and proteins. A special experiment was accordingly carried out in order to ascertain its intracellular distribution in the blue-green mutant. A sample of washed organisms was ground with alumina, and after low-speed centrifugation of the resulting extract to remove alumina and coarse debris, the chromatophores and the soluble fraction were isolated by differential high-speed centrifugation. The amounts of protein, chlorophyll and phytoene in these fractions and in an equivalent sample of the original suspension of organisms were then determined. The recoveries of the fractions from the cell extract were far from quantitative, and hence for purposes of comparison the percentage recovery of each fraction had to be calculated by indirect means. No chlorophyll occurred in the soluble fraction; therefore the recovery of the chromatophore fraction could be estimated by comparing the total chlorophyll content of the isolated chromatophores with the chlorophyll content of the original suspension of organisms. This calculation showed a chromatophore recovery of 16.4 %. By applying the same correction factor to the protein content of the isolated chromatophores (49 mg.), it was calculated that the original total protein content of the chromatophore fraction was 297 mg. Subtracted from the total protein content of the organisms (786 mg.), this gives a value of 489 mg. for the protein of the organisms not in the chromatophores. The total protein content of the isolated soluble fraction was 225 mg., and on the assumption that 'protein not in chromatophores' was all in this fraction, the recovery of the soluble fraction was 45 %. By applying these correction factors for recovery of two cell-fractions to the determined phytoene values, one can obtain an approxi-

mate indication of the intracellular distribution of phytoene (Table 1). The calculated recovery of phytoene was 77% of that present in the whole organisms, and of this, over 90% was in the chromatophore fraction. It must be admitted that the experiment is a highly inaccurate one; however, a second type of calculation substantiated the general conclusion that the phytoene is concentrated in the chromatophores. This is the calculation of the phytoene: protein ratios, shown in Table 2; in whole organisms, this ratio is 2.82. It increases in the chromatophore fraction to 5.10, and decreases in the soluble fraction to 0.31.

Table 1. *Intracellular distribution of phytoene in the blue-green mutant of Rhodopseudomonas spheroides*

Material	Recovery of fraction (%)	Phytoene content (mg.)	Phytoene content, corrected for recovery (mg.)	Phytoene, amount in whole organisms (%)
Whole organisms	100	2.22	2.22	100
Chromatophores	16	0.25	1.54	70
Soluble fraction	45	0.07	0.16	7

Although both calculations show that most of the phytoene is in the chromatophores, a detectable amount was none the less found in the soluble fraction. This may have resulted from a partial disintegration of chromatophores during the preparation or handling of the cell-free extract; but the absence of chlorophyll from the soluble fraction makes this interpretation somewhat unlikely. Accordingly, it is possible that a small part of the phytoene occurs in the organisms unattached to chromatophores.

Table 2. *Phytoene-protein ratios in organisms, chromatophores and soluble fraction of the blue-green mutant of Rhodopseudomonas spheroides*

Material	Phytoene content (mg.)	Protein content (mg.)	Phytoene, mg./g. protein
Whole organisms	2.22	786	2.82
Chromatophores	0.25	49	5.10
Soluble fraction	0.07	225	0.31

Biological observations on the mutants

Green phenotype. Four induced and two spontaneous mutants of this class were isolated. Numerous kinetic experiments have been conducted with one of the spontaneous green mutants (Cohen-Bazire *et al.* 1956), whose growth rate proved to be identical with that of the wild type under all environmental conditions; less detailed observations on the other strains indicate that the same thing may be true for the class as a whole. Accordingly, the change in carotenoid constitution does not seem to affect the gross photosynthetic ability. Most strains of this phenotype are genetically stable when maintained

anaerobically in the light; two strains have shown occasional reversions to the wild type.

Brown phenotype. Three induced mutants of this class were isolated. They grow well under photosynthetic conditions, but systematic comparisons with the growth rate of the wild type have not so far been made. Cultures maintained anaerobically in the light give rise to occasional paler brown mutants, but reversion to the wild type has never been observed.

Dark red phenotype. Numerous induced mutants of this class were isolated, but genetic instability made their maintenance exceedingly difficult. They grow well under photosynthetic conditions, but readily give rise to new mutant types with little or no photosynthetic ability, as well as reverting to wild type.

Blue-green phenotype. Four induced mutants of this class were isolated. They can grow photosynthetically, but the rate is never as great as that of the wild type. Originally all members of the group were very unstable when grown anaerobically in the light, mass reversion to the wild type occurring after one or two transfers. As a result of repeated selection over the past 12 months, one strain has now been stabilized, and can often be carried through several transfers under photosynthetic conditions without reversion. When grown aerobically in the dark, the blue-green mutants do not revert, but tend, sooner or later, to lose their photosynthetic ability as a result of the appearance of weakly pigmented or unpigmented secondary mutants. The safest method of maintenance for mutants of this class is, therefore, cultivation on slopes in the dark, alternated with a periodic transfer through a flask culture incubated in the light (i.e. under photosynthetic growth conditions), followed by streaking and selection of a typical colony, from which a new series of transfers on slopes is initiated.

Photosynthetically-grown cultures of this phenotype have a bright blue-green colour. On aerobic plates, however, the characteristic pigmentation is less easily seen; the colonies are a very pale greyish green, difficult to distinguish without practice from those of colourless mutants. A detailed study of the biology of one of the blue-green mutants is reported elsewhere (Sistrom, Griffiths & Stanier, 1956).

Colourless phenotype. Six induced mutants of this class were isolated. The class is a provisional one, and its designation is somewhat of a misnomer, since the colour of the organisms actually varies from white to a deep tan or brownish green in the different strains included. What is characteristic of all members is the absence of chlorophyll and carotenoid peaks in the spectra of the organisms, coupled with inability to grow photosynthetically. When placed under photosynthetic growth conditions, three strains revert to the wild type. In the absence of reversion, the identity of the remaining three strains as mutants of *Rhodospseudomonas spheroides* is not absolutely certain; but in morphology and colony form they closely resemble the wild type. Biochemical studies, so far not undertaken, may well show that this class includes several different phenotypes.

DISCUSSION

The colourless mutants

Most of the mutant phenotypes isolated from *Rhodopseudomonas spheroides* differ from the wild type with respect to pigmentation only in their carotenoid content. An exception is the group of colourless mutants, which have lost both bacteriochlorophyll and carotenoids. Since some of these mutants revert readily to the wild type, the simultaneous loss of chlorophyll and carotenoids from the organism can presumably take place in a single mutational step. Several possible mechanisms can be envisaged. First, it is conceivable that the ability to elaborate chromatophores or the structure of the chromatophore itself may be affected. Secondly, both classes of photosynthetic pigments may share a common biosynthetic origin, the mutational block in the colourless mutants occurring before the branch point. This interpretation need not necessarily imply a common pathway for porphyrin and carotenoid synthesis, since a common block in the synthesis of carotenoids and of the phytol side-chain of chlorophyll would no doubt be effective in preventing chlorophyll synthesis. The third possibility is that the colourless mutants are blocked genetically in the pathway of chlorophyll synthesis, this entailing as a secondary and indirect consequence the failure of carotenogenesis. It should be recalled that owing to their inability to photosynthesize, these mutants can be grown only in the presence of oxygen, which effectively decreases the synthesis of both chlorophyll and carotenoids in the wild type. Whatever the explanation, these mutants prove that non-photosynthetic variants of the *Athiorhodaceae* can exist, thus answering the question posed in 1944 by van Niel.

The chain of carotenoid synthesis in the photosynthetic mutants

Table 3 summarizes the distribution of carotenoids in the wild type and in the brown, green and blue-green mutants. With the exception of the dark red mutant, which contains both wild-type carotenoids together with small quantities of a new red pigment, all the photosynthetic mutants are characterized by the accumulation of carotenoids which absorb light at shorter wavelengths than do those of the wild type; the extreme instance of this behaviour is shown

Table 3. *The distribution of carotenoids in Rhodopseudomonas spheroides and some of its mutants*

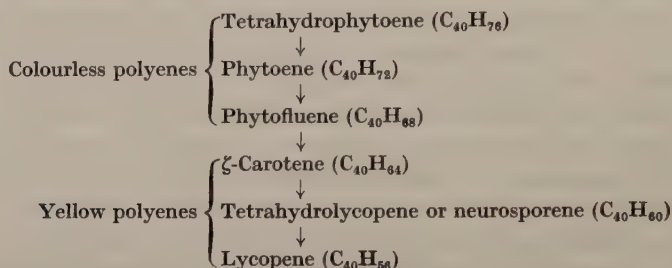
	Phytoene (297 m μ .)*	Mutant yellow (slow)† (466 m μ .)	Mutant yellow (fast)‡ (466 m μ .)	Wild-type Yellow (481 m μ .)	Wild-type Red (512 m μ .)
Wild type	—	—	Trace	+	+
Brown	—	—	+	+	Trace
Green	—	+	+	—	—
Blue-green	+	—	—	—	—

* Figures in parentheses are the values for the longest absorption maximum of the pigment, in light petroleum.

† Dihydroxyneurosporene.

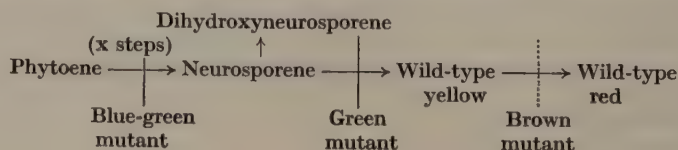
‡ Neurosporene.

by the blue-green mutant, which contains no coloured carotenoids, and accumulates instead phytoene, a colourless polyene whose light absorption occurs exclusively in the ultraviolet region. The shift in spectrum shows that the carotenoids of the mutants contain less extensive systems of conjugated double bonds than do those of the wild type. If we interpret these mutants in the customary fashion, as strains in which the normal pathway of biosynthesis has been blocked at specific points by derangements of the relevant enzyme systems, the results are in general agreement with the mechanism of carotenogenesis proposed by Porter & Lincoln (1950). As a result of their studies on the distribution of carotenoids and colourless polyenes in tomato fruits of different genotypes, these authors postulated that the penultimate stages of carotenoid formation involve stepwise dehydrogenations of originally highly saturated open-chain C_{40} hydrocarbons:



Other carotenoid pigments could be derived from the later members of this series by further dehydrogenation, ring closure and the introduction of oxygen atoms. The Porter-Lincoln series has been greeted with considerable scepticism by other workers on carotenoids (e.g. Mackinney, 1952; Goodwin, 1952, 1955); it remains, however, the only general hypothesis of carotenogenesis so far supported by any evidence. One of the greatest weaknesses of the series as originally postulated was the fact that the structures of its more saturated members were far from established; indeed, later work suggests that phytoene is an octahydrolycopene, and that phytofluene may also be at this oxidation level, differing from phytoene in its conjugation system (see Goodwin, 1955, for a discussion). Thus at present the sequential relationships in the series are far from clear. None the less, the general concept implicit in the Porter-Lincoln series may be correct. Its predictions have been borne out by recent work on mutants of *Chlorella* (Claes, 1954), which showed that blocks in normal carotenogenesis caused accumulations of the more saturated members of the Porter-Lincoln series; and as mentioned above, our findings with *Rhodospseudomonas spheroides* also fit the general concept.

The pattern of distribution of carotenoids in the mutants of *Rhodospseudomonas spheroides* can be explained in terms of the following tentative pathway:



The brown mutant accumulates approximately equal amounts of neurosporene and of the wild-type yellow pigment, together with traces of wild-type red. These facts suggest that it is almost completely blocked in the performance of the normal yellow \rightarrow red conversion, and that the resulting build-up of the normal yellow pigment causes a substantial accumulation of its immediate precursor, neurosporene. This interpretation is supported by the fact that traces of neurosporene occur in the wild type. The very close spectral similarity of the normal yellow pigment to neurosporene indicates that it has a similar structure, and fits the notion of a close biosynthetic relationship.

The green mutant accumulates a mixture of neurosporene and dihydroxyneurosporene, but neither of the wild-type pigments. This fact suggests that it is completely blocked in the conversion of neurosporene to the wild-type yellow pigment. Dihydroxyneurosporene might be either a precursor of neurosporene or a side-product; we have shown it in the latter role.

Lastly, the blue-green mutant, which accumulates exclusively phytoene in quantities roughly equivalent to the quantities of pigments present in wild-type cells, is most easily interpreted as having a complete block in the main pathway of carotenogenesis immediately after phytoene. There are possibly several steps between phytoene and neurosporene; if so, further work should reveal the existence of additional mutant classes with blocks in this region of biosynthesis.

One of the arguments that has been used against the Porter-Lincoln scheme is the failure to detect colourless polyenes such as phytoene or phytofluene in many organisms and tissues which contain carotenoid pigments. However, biosynthetic intermediates may not necessarily be detectable in cell materials which contain the final products; the presence or absence of such intermediates will be governed by the relative rates of the successive biosynthetic step-reactions. The apparent absence of phytoene from cells of the wild type of *Rhodopseudomonas spheroides*, which was reported by Goodwin *et al.* (1955) and has been confirmed by us, is a case in point.

The blue-green mutant constitutes the first known exception to the rule that functional photosynthetic systems always contain carotenoid pigments in addition to chlorophyll (Strain, 1949). Despite the fact that coloured carotenoids have been entirely replaced by phytoene, this mutant is still capable of photosynthetic growth. However, detailed physiological studies (Sistrom *et al.*, 1956) show that it has suffered certain functional derangements.

The absorption spectrum of bacteriochlorophyll in Rhodopseudomonas spheroides

The absorption spectra of bacteriochlorophyll in the green and brown mutants show no detectable changes from that in the organisms of the wild type; but an entirely new pattern of infra-red absorption is found in the blue-green mutant, despite the fact that its chlorophyll is chemically identical with the bacteriochlorophyll of the wild type.

It has long been known that the infra-red spectra of *Rhodopseudomonas*

spheroides and other species of purple bacteria contain certain bands that have no counterparts in the spectrum of isolated bacteriochlorophyll. The comparison of the spectra of bacteriochlorophyll *in vivo* and *in vitro* is complicated by the shifts in the positions of the maxima which occur upon extraction, but on any scheme of assignment, certain of the bands observed *in vivo* remain unaccounted for in the spectrum *in vitro*. Consequently it has been generally assumed that within the organisms bacteriochlorophyll is conjugated with two or more proteins, these several chlorophyll-protein conjugates having different absorption maxima in the infrared region. On this interpretation of the normal infrared spectrum, the disappearance of the peaks at 805 and 855 $m\mu$. in the infrared spectrum of the blue-green mutant would reflect the loss from the chromatophore of two proteins, which are conjugated with chlorophyll in wild-type organisms and the other photosynthetic mutants. Such an explanation cannot be ruled out, although it would necessitate a complex interpretation of the primary biochemical lesion in the blue-green mutant, which also evidently affects carotenogenesis. A more attractive hypothesis is one which assumes that the change of infra-red absorption is a consequence of the elimination of coloured carotenoids from the chromatophore; several possible ways in which such an elimination might affect the infrared spectrum can be envisaged, and are discussed at greater length elsewhere (Calvin, 1955; Sistrom *et al.* 1956).

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Dictyostelium polycephalum n.sp.: a New Cellular Slime Mould with Coremiform Fructifications

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SUMMARY: A unique species of *Dictyostelium* is described for which the binomial *Dictyostelium polycephalum* is proposed because of the seemingly branched character of its fructifications. This slime mould has been isolated repeatedly from samples of surface soil and decomposing leaves collected from deciduous forests in various parts of the United States. As in other members of the Acrasieae, its vegetative phase consists in the independent growth of free-living myxamoebae which feed upon bacterial cells, and its fruiting phase is characterized by the inflowing of these cells to form multicellular organizations preparatory to fructification. *D. polycephalum* differs from previously known species of the genus, particularly for its formation of cell aggregates which give rise to varying numbers of long, thin migrating pseudoplasmodia, and for the potential capacity of each of these to form subsequently a small coremiform fructification consisting of multiple sorocarps. Whereas some simple sorocarps are regularly produced, fructifications under optimal conditions typically consist of from two to ten adherent sorocarps. In such coremiform structures, the sorophores of individual sorocarps, although clearly distinguishable microscopically, are tightly appressed for approximately three-quarters of their length, at which level they diverge sharply and at their apices bear globose spore heads, or sori. The spores are elliptical to reniform as in most species, but germinate by the swelling and dissolution of the spore wall in a median plane rather than by longitudinal splitting.

The genus *Dictyostelium*, established by Brefeld with his description of *D. mucoroides* in 1869, is characterized by simple or irregularly branched fruiting structures, or sorocarps. As in all of the simple cellular slime moulds of the class Acrasieae, the vegetative stage consists in the growth and multiplication of small free-living amoeboid cells, or myxamoebae. In contrast, the fruiting stage is characterized by the aggregation of these myxamoebae into multicellular organizations, termed pseudoplasmodia, followed by their progressive integration and differentiation to form either structural elements of the sterile supportive stalk, or sorophore, or reproductive cells, spores, which with enveloping slime comprise the spore head, or sorus.

Species of *Dictyostelium* are widely distributed in nature and occur with generally unappreciated frequency in persistent accumulations of well-rotted leaf mould in deciduous forests. Less than a dozen species have been described. Of these, only six are in any sense well known: *D. mucoroides*, very abundant and very variable, is characterized by milk-white sori and generally unbranched, or sparsely branched, sorophores. *D. purpureum* Olive (1901, 1902), less commonly encountered, differs from the preceding primarily in producing deep purple sori. *D. minutum* Raper (1941*b*) represents a consistently diminutive form, but otherwise resembles *D. mucoroides*. *D. lacteum* Van Tieghem (1880), relatively rare and only recently rediscovered (Raper, 1951), is an

extremely delicate species characterized by spherical spores. *D. giganteum* Singh (1947) is a robust species doubtfully separable from *D. mucoroides*. *D. discoideum* Raper (1935) is characterized by migrating pseudoplasmodia and by erect tapering sorophores that arise from flattened basal disks.

A cellular slime mould differing markedly from any of the above and from any previously described form has been isolated repeatedly during the past five years, and hence merits recognition as a new species. It typically exhibits a migrating pseudoplasmodial stage, thus suggesting *Dictyostelium discoideum*. Aside from this single character, however, it bears little resemblance to that species. It is distinguished particularly by its coremiform fruiting structures which consist of a variable number of sorocarps. In such compound structures the individual sorophores are easily discernible, these being parallel and closely adherent throughout approximately three-quarters of their total length, then strongly divergent with each terminated by a small globose sorus. Considered individually, the clustered sorocarps would seem to represent a diminutive and simple *Dictyostelium*; viewed under low magnifications, the coremiform clusters appear as single, well-proportioned structures sometimes bearing as many as 8 to 10 spore heads. Because of these equally important characteristics, the species is placed in the genus *Dictyostelium* and the binomial *D. polycephalum* is proposed for it. A technical description of the newly discovered slime mould, together with brief accounts of its isolation, cultivation, and the more salient features of its developmental history follows:

TECHNICAL DESCRIPTION

Dictyostelium polycephalum sp.nov.

Cultum ad 25–30 C in substrato solido (agar-agar), dilutam faeni infusionem vel dictam 'glucose + peptone' solutionem continente, cum diversis bacteriis fungisque mixtum, praecipue cum *Aerobacter aerogenes* et *Dematium nigrum*. Sorocarpi plerumque 375–650 μ . alti, seape singuli sed typice plures usque ad 10 in coremiformen fructificationem fasciculati. Sorophori per maiorem longitudinis partem inter se conjuncti, sed apices versus disjuncti. Sori albi vel hyalini, globosi, 45–90 μ . diam. Sporae ellipticae vel reniformes, plerumque 6.0–7.5 μ . longae, 3.0–3.5 μ . latae.

Hab. Ex foliis putrescentibus et summo solo in deciduis silvis isolatum, South Carolina, Texas, Michigan, Wisconsin et Illinois, U.S.A. (Strain S-4, Maio, 1951 isolatum typus).

Cultures grown upon dilute hay-infusion or dilute glucose + peptone agars in association with a mixed bacterial and fungus flora at temperatures between 25 and 30°; sorocarps small, often simple but typically clustered to form coremiform fructifications consisting of variable numbers of sorocarps, commonly 375–650 μ . in height, with sorophores appressed throughout most of their length, diverging terminally to bear white to colourless, globose sori 45–90 μ . in diameter; spores elliptical to reniform, usually 6.0–7.5 \times 3.0–3.5 μ .; myxamoebae form aggregates typical of the group, but these give rise to several migrating pseudoplasmodia which in turn may produce either simple sorocarps or compound fructifications consisting of several sorocarps.

Isolated from decaying leaves and surface soil from deciduous forests, South Carolina, Texas, Michigan, Wisconsin and Illinois, U.S.A.

ISOLATION AND CULTIVATION

The type culture of *Dictyostelium polycephalum*, strain no. S-4, was isolated in May 1951 by the writer and Miss Dorothy Fennell at the Northern Regional Research Laboratory, Peoria, Illinois, U.S.A., from a sample of well-rotted leaf mould collected from a predominantly hardwood forest near Holly Hill, South Carolina. The principal vegetation at the site of the collection consisted of live oaks, maples, sweet gum, and a variety of other deciduous trees and shrubs. The slime mould was isolated in the conventional way. A small portion of the sample (c. 1.0 g.) was added to approximately 10 vol. of sterile water, shaken vigorously, and the resulting suspension streaked upon plates of hay-infusion and dilute hay-infusion agars (Raper, 1937, 1951). *Dictyostelium polycephalum* was first observed several days later as diminutive fruiting structures bearing from one to five small glistening spore masses. It was hardly suggestive of a simple slime mould, but neither did it suggest any fungus with which we were familiar. Microscopic examination of the supporting stalks, composed of tiers of superimposed vacuolated cells, quickly revealed its true relationships. Isolations were made by the routine procedure of planting spores at the intersection of crossed streaks of *Escherichia coli* on the media cited above. The myxamoebae grew satisfactorily and the streaks of *E. coli* were completely obliterated. The myxamoebae were observed to collect into rounded clumps from which emerged thin and variously contorted migrating pseudoplasmodia. Scattered fruiting structures were subsequently produced and these appeared, for the most part, in areas characterized by appreciable contamination with other bacteria and fungi introduced with the spore inoculum. By repeated transfer the slime mould was obtained in two-membered culture with *E. coli* but few sorocarps were produced, and these usually consisted of simple sorophores terminated by single sori. Meanwhile, parallel cultures inoculated with the mixed microflora originally associated with the slime mould continued to exhibit more complex fruiting structures in limited numbers, some of these bearing as many as 5 or 6 sori. It seemed probable that the new species might require unusual cultural conditions for optimal growth and development.

Utilizing the two-membered culture with *Escherichia coli*, cultivation of the slime mould was attempted upon a variety of culture media. Substrata containing relatively low concentrations (c. 0.1 %, w/v) of glucose or lactose as a carbon source and peptone or yeast extract as a source of nitrogen supported a fair growth of the associated bacteria and permitted the slime mould to vegetate in an apparently normal if not luxuriant manner. Media containing greater concentrations of such nutrients, particularly peptone or yeast extract, were generally less satisfactory, and in no case did the slime mould develop optimally beyond the vegetative stage or an early fruiting stage characterized by the formation of abundant migrating pseudoplasmodia. Such media were not improved materially by the addition of phosphate buffers (0.01 M or 0.02 M) as previously used for other members of the group (Raper, 1939, 1951). Fruiting structures, when present, rarely exceeded in complexity the simple

structures previously observed with *E. coli* on the hay-infusion agars. Trials with other bacterial associates, including *Aerobacter aerogenes*, *Serratia marcescens* and *Pseudomonas fluorescens*, were equally unrewarding. It may be noted that in association with *Serratia marcescens* the sori of the slime mould remained uncoloured, hence resembling *Dictyostelium mucoroides* and *D. minutum* rather than *D. discoideum* in this regard.

By accident a plate culture of *Dictyostelium polycephalum* growing in association with *Aerobacter aerogenes* on dilute hay agar became contaminated with *Dematium nigrum*,* and in the area adjacent to this yeast-like fungus numerous well-formed sorocarps developed. Attention was immediately directed toward growing the slime mould in 3-membered cultures with *A. aerogenes* and *Dematium nigrum* upon a wide variety of substrata, including various modifications of thin hay agar and media containing relatively low but varying concentrations of glucose or lactose as a carbon source and peptone or yeast extract as a source of nitrogen. Of many combinations examined, maximal development of fruiting structures was obtained upon a medium that contained 0.5 % glucose and 0.1 % peptone solidified with 2 % agar, and designated '0.5 D-0.1 P'. In three-membered cultures of this type incubated at 24-26°, growth of the slime mould was comparatively slow. Migrating pseudoplasmodia first appeared after 4-6 days and continued to form over a period of 2-3 weeks. Sorocarp formation was initiated generally after 10-14 days and continued for an additional 2-3 weeks. Pseudoplasmodial migration was often quite extensive and the coremiform fruiting structures were generally formed throughout the Petri plate with only limited concentration in zones adjacent to the sites of inoculation. A portion of such a culture is illustrated in Pl. 1, fig. 1, whereas pseudoplasmodia and mature fructifications formed midway between two parallel streaks seeded with the three organisms are shown, substantially enlarged, in Pl. 1, fig. 2. Equally satisfactory cultures were obtained either by streaking a mixed inoculum of the three micro-organisms on the agar plates, or by first cross-streaking the bacteria and the fungus and subsequently implanting spores or myxamoebae of the slime mould at the intersection of such streaks.

The particular advantage afforded by growing *Dictyostelium polycephalum* in a three-membered culture on 0.5 D-0.1 P is not immediately apparent. However, it is obvious that each of the associated micro-organisms makes its own beneficial contribution to the microcosmos wherein the slime mould thrives. In the absence of the bacteria, no apparent growth of *D. polycephalum* occurs. In the absence of the fungus, satisfactory growth of myxamoebae is readily attained and wheel-shaped aggregates commonly develop, followed by the formation of numerous migrating pseudoplasmodia. These latter structures, however, often fail to produce mature fructifications, and at most develop slight structures bearing one, two or rarely three sori. The presence of the fungus, however, in some manner renders the culture more favourable for slime mould growth; more specifically, it permits fructification of

* Identification by Dr L. J. Wickerham, Northern Utilization Research Laboratory, Peoria, Illinois.

D. polycephalum at the optimal level thus far attained. Certain explanations suggest themselves: (1) the fungus may produce some metabolite which favours sorocarp formation; (2) it may remove or neutralize some inhibitory substance(s) produced by the growing bacteria; (3) it may alter the physical environment in which the slime mould grows; or (4) it may effect a combination of these functions, thus promoting fructification. These possibilities are now being investigated with my associate, Dr W. F. Whittingham.

Cultural conditions favouring normal fructification in any of the Acrasieae are more exacting than those required for satisfactory growth (Raper, 1956). However, in *Dictyostelium polycephalum* this disparity seems to be particularly accentuated. Many substrata have been employed, whereon seemingly satisfactory or even rich growth of this slime mould has been obtained followed by sparse, incomplete or aberrant fructification. Whereas such cultures leave much to be desired, they have provided a useful and, it is believed, generally accurate picture of the aggregating and migrating pseudoplasmodial stages of this species. On 0.5D-0.1P in the presence of either *Escherichia coli* or *Aerobacter aerogenes* as the sole associate, the slime mould grows quite well at room temperature and develops aggregating pseudoplasmodia which subsequently give rise to numerous migrating bodies. However, the aggregating pseudoplasmodia in such cases are generally quite dense and of restricted dimensions, hence it is often difficult to detect the earliest stages of aggregation or to follow the inflowing streams of myxamoebae. Media containing lactose and yeast extract have proved especially useful for such observations and a formulation containing 0.5 % lactose, 0.05 % yeast extract and 2 % agar is especially recommended (Pl. 2, figs. 3-5 and 9). Greater or lesser growth can be obtained by increasing the nutrient content to 1.0 % lactose and 0.1 % yeast extract or by decreasing it to 0.05 % lactose and 0.01 % yeast extract, respectively. In none of these cases, however, does optimal fruiting ensue, and fructifications, when formed, consist of small structures bearing one, two or rarely more sori. On the other hand, well-proportioned migrating pseudoplasmodia regularly develop in numbers that reflect the richness or paucity of myxamoebic growth.

The optimum temperature for *Dictyostelium polycephalum* appears to be about 30°, which is somewhat higher than that for *D. discoideum* and other species of the Dictyosteliaceae previously investigated by the writer. Excellent but less rapid growth can be obtained at lower temperatures, with migrating pseudoplasmodia first appearing after 3 weeks at 15°. The maximum temperature for the species appears to be in the neighbourhood of 34-35°.

The optimum reaction for *Dictyostelium polycephalum* appears to be approximately pH 6.5, although excellent growth of myxamoebae and the formation of abundant migrating pseudoplasmodia have been observed within the range pH 5.5-7.2. Growth is limited and few pseudoplasmodia develop at pH 5.0, and at pH 4.5 growth is commonly lacking. Numbers of pseudoplasmodia decrease and the pattern of those which are formed becomes increasingly atypical as the pH value rises above 7.5. The pH value attained by any culture is strongly influenced by the composition of the substrate and the temperature

of incubation; hence cognizance of this interdependence is essential for the successful cultivation of this slime mould, as it is for other species investigated previously. This becomes particularly important at temperatures exceeding those of the laboratory, namely *c.* 24–26°.

Additional strains of *Dictyostelium polycephalum* have been isolated from samples of soil collected from deciduous forests in Texas, Wisconsin, Michigan and Illinois. The species is not particularly rare, and in some collections it is quite commonplace. For example, it was isolated from seven of twenty-five samples of leaf mould and surface soil collected in September 1954 from a virginal deciduous forest at Funk's Grove, Illinois. All of these isolates have been grown in two-membered cultures in association with *Escherichia coli* or *Aerobacter aerogenes* upon most of the aforementioned media, and none of them seem to differ very markedly from the type strain, no. S-4, upon which the following account of the developmental cycle of the new species is centred.

GROWTH AND MORPHOGENESIS

Spore germination

The spores of *Dictyostelium polycephalum*, as in most other members of the Acrasieae, are capsule-shaped to somewhat reniform. They are quite variable in size, usually ranging from 6.5–7.0 × 3.0–3.5 μ . but with some cells measuring as much as 8.0 × 3.5 μ . and others as little as 4.0–4.5 × 2.5 μ . Germination in this species is rather different from that previously observed. The spore case does not split longitudinally at one end to release an amoeboid cell. Instead, the protoplast swells and the spore wall is bulged out in a median area, becoming noticeably thinner all around this region of the spore. Eventually the wall bursts or is completely dissolved and the protoplast slowly emerges. During germination the two ends of the empty spore case may be joined at one side by a tenuous membrane, or they may become completely separated. The process is often completed within 1 hr.

Vegetative growth

Upon germination the myxamoeba of *Dictyostelium polycephalum* begins to feed upon the bacteria adjacent to it, enlarges and divides. As in other species, the vegetative stage consists in the independent growth and repeated division of such daughter cells until a large population of cells is built up and the available food supply is consumed, at which time the fruiting stage is initiated by the formation of aggregating pseudoplasmodia. Nothing that would distinguish these myxamoebae from those of other Dictyostelia has been observed. As seen growing on an agar surface they are very irregular in shape and size and range from broadly triangular or elongate to more or less rounded (Pl. 2, fig. 7). They may or may not exhibit prominent pseudopodia. Posteriorly placed contractile vacuoles are conspicuous and a single myxamoeba may show two or three of these which normally discharge as one but may empty independently. The nucleus is difficultly discernible in unstained cells. A zone of clear ectoplasm can be seen anteriorly in cells that are rapidly moving and

in those having extended pseudopodia. The finely granular endoplasm, comprising the bulk of the myxamoebae, shows numerous food vacuoles containing bacteria in varying stages of digestion. Whereas linear dimensions in amoeboid cells must be interpreted with caution, size ranges and proportions have some descriptive significance, and in *D. polycephalum* the vegetating myxamoebae commonly range from $5.0-7.5 \times 8.0-12.0 \mu$. with seemingly larger and smaller cells not uncommon.

Under optimal conditions for growth and development, the vast majority of the vegetative myxamoebae aggregate to form pseudoplasmodia at the close of the vegetative stage and eventually these, or their daughter cells, differentiate to form the structural elements, stalk cells and spores, of mature sorocarps. However, in most cultures limited numbers of more or less isolated myxamoebae fail to enter the wheel-like aggregates and instead round up as individual cells, become encased by somewhat rigid cellulose walls to form microcysts, and in this condition enter a temporary to prolonged resting stage. In certain cultures which are suboptimal for one reason or another, the greater portion of the free-living vegetative population forms microcysts and never enters the normal fruiting process. This phenomenon is often particularly marked in cultures grown with *Aerobacter aerogenes* on substrates containing lactose and yeast extract, and seemingly it is most pronounced in the more central areas of broad bands of mixed bacteria and slime mould growth. Individually the microcysts are spherical or nearly so, measure approximately $4.0-6.5 \mu$. and germinate by a simple process of excystment whereby the wall of the microcyst splits open and the amoeboid protoplast emerges to renew its vegetative cycle.

Pseudoplasmodium formation

The phenomenon of myxamoebic aggregation, or pseudoplasmodium formation, in *Dictyostelium polycephalum* is basically similar to that in species already known. With the exhaustion of the available food supply (bacteria) the myxamoebae elongate, become uniformly oriented and stream toward centres of aggregation presumably in response to some chemotactic stimulus, as in *D. discoideum* (Bonner, 1947; 1949), which is first produced by the centrally placed myxamoebae and subsequently by those comprising the radiating arms of inflowing cells. During this process the myxamoebae crowd together and assume a characteristic limax form, their dimensions commonly ranging from $6-4 \times 15-20 \mu$. (Pl. 2, fig. 8). Such aggregating cells are characterized by conspicuous anterior zones of non-granular ectoplasm, inconspicuous food vacuoles, and contractile vacuoles which continue their rhythmic enlargement and discharge. In contrast to *D. discoideum*, *D. mucoroides* and *D. purpureum*, where conspicuous radiating streams usually develop soon after the centres of aggregation become apparent, the developing pseudoplasmodia of *D. polycephalum* may for several hours remain evident only as areas of somewhat greater cell concentration surrounding small but definitely raised centres. Superficially, the extent of the nascent aggregate may be indicated by a more or less well-defined halo as seen in Pl. 2, fig. 3. During this stage, and for

some time thereafter, the myxamoebae normally continue to aggregate as broad and nearly continuous, sheets of inflowing cells that more or less completely surround the point of aggregation. As aggregation progresses, prominent and characteristic wheel-like patterns usually emerge, with the radiating streams often tending to terminate abruptly, as seen in Pl. 2, fig. 4. In other cases, discrete streams are more sharply defined and are strongly anastomosed (Pl. 2, fig. 5), whereas in still others the pseudoplasmodia may persist for a longer time as broad inflowing streams which at times exhibit concentric, or transverse, bands of alternately crowded and thinly disposed cells, probably indicating a wave-like progression of the aggregating cells (Pl. 2, fig. 6). The developing pseudoplasmodia commonly attain a diameter of 5–7 mm. As aggregation proceeds, the central area becomes papillate and from each of the papillae arises a long thin upright column of myxamoebae. These columns continue to develop simultaneously with the influx of myxamoebae from the peripheral areas of the aggregates, and in time become several millimetres in length, topple over so that they rest on the surface of the agar and begin a slow migration outward from the centres where they were formed (Pl. 2, fig. 9).

Migrating pseudoplasmodia

The migrating pseudoplasmodia of *Dictyostelium polycephalum* must, I think, be considered comparable to those produced in *D. discoideum* (Raper, 1935, *et seq.*); nevertheless, they differ from the latter in a number of significant particulars. First, as many as a dozen or more migrating bodies may arise from a single aggregation centre. Secondly, they are, by comparison, quite long and very thin, exhibit no apparent response to light or temperature, and show a markedly greater tendency to become transversely segmented. Thirdly, no migrating pseudoplasmodium has ever been observed to divide dichotomously nor have two of these structures been seen to coalesce, thereby forming either a smaller or larger, but otherwise normal, fruiting organization as in *D. discoideum*. Finally, each migrating body is potentially capable of producing a number of sorocarps which develop simultaneously and together constitute a coremiform fructification, whereas in *D. discoideum* a single migrating body, of whatever origin, typically gives rise to a single sorocarp (Raper, 1940*a*; 1941*a*).

The migrating pseudoplasmodia of *Dictyostelium polycephalum* merit further consideration. These bodies commonly attain a length of 5–10 mm. or more, while they seldom exceed 50–60 μ . in diameter. They range from almost straight to irregularly curved and twisted in form (Pl. 3, figs. 10, 11), depending in part at least upon the relative rates of progression in different areas of the migrating masses. They may be essentially uniform in diameter throughout, or they may show relatively thick and thin areas, and in extreme cases may present a strikingly beaded appearance, the significance of which is not at present known. Migration presents some interesting problems. It is not uncommon to observe a migrating pseudoplasmodium which is in contact with the agar only at its posterior end, or to see an elongate body which touches the substrate at only two or three points throughout its entire length, or again to see one moving

through the aerial hyphae of some accompanying fungus as illustrated in Pl. 3, fig. 12, where a species of *Absidia* has been introduced into the culture vessel. When moving on agar the elongate pseudoplasmodium commonly becomes thickened at points of contact with the substrate, the enveloping slime sheath becomes ruptured, and the migrating body becomes severed at these sites. The intact portion anterior to such a break will normally continue its migration uninterrupted. The posterior portion may develop a new apical tip and resume migration, or the disintegrative process may continue further with the myxamoebae separating from each other. In the presence of bacteria these will return to the vegetative stage, and in their absence they may or may not reorganize into new and smaller fruiting organizations. The internal organization of the migrating pseudoplasmodium is obviously less highly developed than in *Dictyostelium discoideum*. Whereas careful histological studies still remain to be made, there is no evidence to indicate areas of presumptive specialization into pre-stalk and pre-spore myxamoebae, a condition that is not surprising when one recalls that each of these structures is potentially capable of subsequently subdividing to produce several sorogens. Viewed microscopically, only the myxamoebae that comprise a minute anterior fraction ($c. 50 \pm \mu.$) exhibit a transverse orientation.

Under optimal conditions the migrating pseudoplasmodia may travel for several centimetres before halting to form sorocarps. The rate of migration is generally about 0.5 mm./hr. in contrast to 1.0–2.0 mm./hr. for *Dictyostelium discoideum* (Raper, 1940a).

The migrating pseudoplasmodia of *Dictyostelium polycephalum* appear to exhibit little or no response to light. This is in marked contrast to *D. discoideum* where light is a principal stimulus governing pseudoplasmodial migration, and to *D. mucoroides*, *D. purpureum* and *Polyspondylium violaceum* where light so strikingly determines the length of the sorophores and the direction in which sorocarps are built (Raper, 1940a, b, 1941a).

Sorocarp formation

Following varying periods and distances of migration the pseudoplasmodia cease forward movement and collect into compact masses preparatory to sorocarp formation. Typically, such a mass gradually invaginates vertically into a number of contiguous but more or less well-defined columns of myxamoebae, each of which will normally build an individual sorocarp. The details of this process have not yet been investigated carefully; nevertheless, sufficient observations have been made to follow the main course of events. Stalk formation is initiated within each of the columns prior to any obvious elongation of these, and as this proceeds the individual sorogens become apparent as they are slowly raised above the substratum (Pl. 4, fig. 13). With the continued elongation of the individual sorophores, the sorogens, or columns of undifferentiated myxamoebae, become increasingly distinct and tend to diverge from one another as seen in Pl. 4, fig. 14. During this period an individual sorophore is being formed within the terminal area of each of these, and yet the processes of cellular differentiation within the several sorogens is sufficiently inter-

dependent so that all of the sorophores are constructed at a nearly uniform rate. It is at present a noteworthy and perplexing fact that whereas the sorophores are lengthening at divergent sites, the completed stalks are in the majority of cases closely appressed and to some degree cemented together to form a well-proportioned, multi-stalked support. This picture changes abruptly when the sorophores reach 75–80 % of their ultimate length, for beyond this stage they cease to be drawn together and each retains its original orientation until sorophore formation is completed. The capsule-shaped spores are produced coincident with this latter stage of stalk formation, and each sorophore terminates in a globose sorus of dimensions proportional to the mass of myxamoebae contributing to its formation. The net result of the morphogenetic changes just outlined is the construction of compound fruiting structures, which consist of a variable number of distinct but conjoined sorocarps. The general habit of such fructifications is shown in Pl. 1, fig. 2, and in Pl. 4, fig. 16; details of their cellular structure can be seen in Pl. 5, fig. 20. Such coremiform fruiting structures may contain as many as eight or ten individual sorocarps, although structures containing from two to six are much more commonplace, and in every culture, however favourable, numerous sorocarps bearing single sori are regularly formed. Except for the absence of a basal disk, such simple sorocarps bear a striking resemblance to the diminutive but otherwise normal fruiting structures found in very thin cultures of *Dictyostelium discoideum*. When reduced to its simplest terms, the formation of compound fruiting structures in *D. polycephalum* may thus be interpreted as the co-ordinated and contiguous development of two or more small unbranched sorocarps.

Microscopic examination readily reveals that the polycephalic fruiting structures of *Dictyostelium polycephalum* represent clusters of discrete sorocarps, and this is clearly shown in Pl. 5. It will be noted that the individual sorophores consist, for the most part, of single tiers of superimposed and strongly vacuolated cells, a structural pattern which is duplicated by other species of *Dictyostelium* in the construction of very small sorocarps. What distinguishes *D. polycephalum* particularly is this: when more myxamoebae are present in a migrating pseudoplasmodium than can be used for the construction of a single sorocarp of the limited dimensions that characterize the species, that fruiting organization responds not by building a larger sorocarp but by dividing into a variable number of parts, each of which builds its own sorocarp of these same general dimensions. Significantly, this concurrent building of multiple sorocarps is far from haphazard. The processes of pseudoplasmodial invagination and subsequent cellular differentiation obviously reflect a considerable measure of community control, otherwise the component parts would not develop at uniform rates, the several stalks would not adhere together, and the constituent sorocarps would be expected to show a much greater diversity in form and size. That a very considerable degree of uniformity exists among the sorocarps that comprise a particular coremiform structure, and to a lesser degree between these and the sorocarps of other compound fructifications, is indicated in Table 1. In this representative tabulation

certain selected measurements were recorded for a number of fructifications of varying complexity observed in a small area of a favourable culture of the type illustrated in Pl. 1. Two simple sorocarps with single sori are included for comparison. The overall height of these fructifications varied only from 350 to 650 μ , with extremes of 40 and 90 μ , being occasionally observed in the diameters of their sori. It is regarded as particularly significant that the length

Table 1. *Proportions of the coremiform fructifications of Dictyostelium polycephalum*

Selected measurements of representative fructifications composed of different numbers of sorocarps produced in a limited area of a three-membered culture of *Dictyostelium polycephalum* grown in association with *Aerobacter aerogenes* and *Dematium nigrum* on an agar medium containing 0.5 % + 0.1 % peptone. Incubation was at c. 24–26° for 3 weeks.

Overall height of fructification (μ .)	No. per fructification	Sori Diameters of individual sori (μ .)	Dimensions of compound stalk			
			Dia- meter at base (μ .)	Dia- meter at mid- point (μ .)	Length: base to diver- gence (μ .)	Length: divergence to termini (μ .)
400	1	75	20	10	—	None
450	1	90	20	12	—	None
475	2	45, 70	18	12	375	75–125
350	2	60, 65	20	15	275	75–100
550	3	50, 60, 60	40	20	425	100–125
500	3	65, 60, 65	25	12	400	75–100
475	3	50, 45, 50	25	15	375	90–100
375	3	40, 45, 45	15	12	275	90–100
500	3	80, 70, 65	30	15	350	125–150
500	4	45, 50, 50, 55	35	15	400	90–100
625	4	90, 80, 80, 75	30	20	475	125–150
550	4	70, 75, 70, 85	35	20	400	110–150
650	5	60, 75, 60, 70	40	25	500	125–150
550	5	40, 40, 50, 60, 45	45	25	450	90–100
575	5	80, 70, 80, 85, 65	45	30	425	125–150
525	6	60, 60, 55, 60, 45, 50	30	15	400	100–125
600	7	70, 60, 70, 65, 75, 55, 60	50	30	475	100–150
650	7	50, 50, 55, 60, 55, 60, 55	60	30	500	125–150
550	9	50, 45, 40, 45, 45, 50, 60, 40, 45	55	30	400	125–150

of the sorophores from the points of divergence to their apices generally represented approximately $20-25 \pm \%$ of their total length. It can hardly be doubted that this consistent proportion reflects a considerable degree of heritable specificity in the control of the morphogenetic processes.

Considered individually, the sorophores show an overall and generally consistent taper from their bases to a site just below the level of divergence, and this is reflected in the corresponding diameter of the compound stem. At their bases the larger sorophores may show two or even three cells in cross-section whereas at their mid-points they usually consist of single rows of cells, and in more terminal areas this is almost invariably the case. Occasionally, one or

more of the sorophores that comprise a coremiform support may be initiated as completely independent entities (Pl. 5, fig. 19). However, as sorophore formation progresses these become increasingly appressed to form a quite rigid unitary support. In the subterminal area of divergence the sorophores thicken substantially, and at this level the diameter of one of these may approach that of the whole bundle at a somewhat lower level. These differences result from the varied proportions assumed by constituent vacuolated cells at different stages in the morphogenesis of the compound fructification, and they can be assumed to reflect a response to the greater loads which must be supported by the individual sorophores as they develop outward from a previously common vertical axis.

DISCUSSION

Dictyostelium polycephalum presents many interesting and unique features in its fruiting phase. The sorocarps are quite diminutive, rarely exceeding $650\ \mu$. in length, and considered individually they are somewhat smaller than those normally formed by any other known member of the genus. Viewed as coremiform clusters, in which form they characteristically occur, they appear to be branched at a single point some distance below the level of the sori. This is in marked contrast to all other species of *Dictyostelium* where branching is either lacking or occurs in an irregular and haphazard fashion, and upon this basis alone one might question the wisdom of placing the new slime mould in this genus. However, when viewed microscopically, the apparent unitary fructification is seen to consist of a number of discrete sorocarps, i.e. individual fruiting structures, the greater portion of whose sorophores are closely adherent but whose sorus-bearing apices are strongly divergent. Comparable fructifications are not found in any other described member of the Acrasieae, although structures somewhat suggestive of them were apparently observed in two genera, *Acrasis* and *Coenonia*, described by van Tieghem in 1880 and 1884, respectively. Whereas he published no illustrations and neither genus has been since reported, his observations on the life cycles of the Acrasieae were most penetrating and his descriptive accounts are sufficiently clear to leave little doubt concerning the overall aspect of the slime moulds that he studied. *Acrasis* was described as sometimes showing a coremiform habit, but the constituent stalks bore chains of spherical and slightly roughened spores rather than sori containing smooth-walled spores of the type found in other forms. The second genus, *Coenonia*, was reported as producing branched fruiting structures when grown in rich culture and, if my interpretation is correct, the branches generally arose at a single level on the upright sorophore. Such a structure, viewed as a whole, might conceivably resemble the compound fructifications of *Dictyostelium polycephalum*, although there is nothing in van Tieghem's account to indicate a coremiform support. The overall dimensions of van Tieghem's *Coenonia* were considerably greater than those reported for the new slime mould, and each branch was reported to terminate in a cupule which supported and partially enclosed a gelatinous mass of spores. Obviously, *Dictyostelium polycephalum* cannot be regarded as closely related to either of

these long-missing slime moulds, but it is believed significant that in its fructifying processes it does exhibit morphogenetic manifestations which are in some measure remindful of van Tieghem's genera. Branched fructifications constitute, of course, the chief distinguishing characteristic of the ubiquitous genus *Polysphondylium* (Brefeld, 1884; Harper, 1929). In this case the fructification unquestionably represents a single unitary sorocarp, just as is believed to be the case in *Coenonia*. In *Polysphondylium* the rising sorogen repeatedly casts off and leaves behind on the lengthening sorophore small masses of myxamoebae which subsequently segment, giving rise to whorls of lateral branches that are anchored to the sorophore and attain maturity coincident with the continued construction and elongation of the main axis. In *Coenonia* the branching habit is suspected of resulting from the segmentation of a single mass of undifferentiated myxamoebae midway during the process of sorocarp construction. The characteristic branching of *Polysphondylium*, and presumably of *Coenonia* as well, is then in no true sense comparable with the pseudo-branching which is such a distinguishing feature of *Dictyostelium polycephalum*, for in the latter case vertical segmentation of the pseudoplasmodial body occurs soon after it ceases migration and precedes any beginnings of sorocarp formation.

Certain other species of *Dictyostelium* regularly produce a variable number of sorocarps from a single aggregating pseudoplasmodium. This is particularly true of the other diminutive species, *D. minutum* and *D. lacteum*, and to a lesser degree in the larger members of this genus and in *Polysphondylium* as well. No other species is known, however, where the sorophores of two or more individual sorocarps are normally adherent. In *Dictyostelium minutum* it is not uncommon to observe a half-dozen or more sorocarps to arise from a single aggregate, and in *D. lacteum* this number is often exceeded. The formation of such sorocarps is regularly initiated while myxamoebic aggregation is still in progress, and each sorocarp obviously develops independently of all the others despite their common origin from a single fractionating aggregate. The gregarious sorocarps arising from a primary pseudoplasmodium in these species thus represent a group, or cluster, of fructifications in which the morphogenetic changes leading to culmination are completely independent in the different sorocarps. The situation in *D. polycephalum* is quite different from this: (1) a migrating stage, which is lacking from these other species, is regularly interposed between the stages of aggregation and sorocarp formation; (2) upon the cessation of migration the pseudoplasmodial mass segments more or less equally along vertical planes prior to the initiation of sorophore formation; (3) these nascent sorogens are basically separate from one another but are contiguous and remain interdependent so that culmination tends to proceed uniformly in all of them; (4) the sorophores which they form are constructed so that they constitute a single, rigid supporting column until they have attained approximately three-quarters of their ultimate height. Only in *Acrasis*, and possibly in *Coenonia*, among the reported cellular slime moulds does an even remotely similar pattern of morphogenesis occur, and for these genera the surmised parallelism in behaviour is highly speculative, for

the completed fructifications in both genera differ markedly in other particulars from those of *Dictyostelium polycephalum*.

A migrating pseudoplasmodial stage regularly occurs in only one other species of the Acrasieae, *Dictyostelium discoideum*, and between this and the migrating pseudoplasmodium of *D. polycephalum* marked differences occur in form, in structure, and in behaviour. Whereas more than one migrating body can and often does arise from a single aggregating pseudoplasmodium in *D. discoideum*, the more usual behaviour is for such a wheel-like aggregate to produce a single, motile, slug-like body and for this in turn to build a single sorocarp. An individual slug may split longitudinally to produce two smaller but otherwise typical migrating bodies, or two migrating structures of separate origins may collide and fuse to form a larger and equally typical migrating pseudoplasmodium, and in either case the migrating pseudoplasmodium regularly forms a single sorocarp of characteristic form and of dimensions proportional to the mass of myxamoebae present in the organization at the time of culmination (Raper, 1940*a*). The migrating pseudoplasmodia of *D. discoideum* show a strong axial polarity and exhibit striking and highly sensitive responses to light and temperature. In contrast, the aggregating pseudoplasmodium of *D. polycephalum* regularly gives rise to many migrating bodies, each of which is potentially capable of producing a coremiform fructification consisting of several sorocarps. In so far as we have observed, the migrating bodies of this species neither split nor fuse to form larger or smaller migrating units.

The form and structure of the migrating structures in the two species are quite different. Whereas the proportions of length to width of the slugs in *Dictyostelium discoideum* vary in response to cultural conditions and other factors, one seldom observes actively moving bodies where this proportion exceeds 10 : 1, while it is commonplace to see migrating bodies of *D. polycephalum* where the same proportions are 100 : 1, or even greater. By comparison, the migrating bodies of the latter species are very long and thin. As in *D. discoideum* they are bounded by an envelope of slime, termed the slime sheath, that is secreted by the mass of myxamoebae, and during migration this is deposited on the agar surface as a continuous hyaline slime track. The envelope is obviously very tenuous and the track is extremely delicate. Possibly the insubstantial nature of the slime sheath of *D. polycephalum* accounts in part for the very elongate form typically assumed by the migrating bodies; certainly it could account for the marked tendency of the slugs of this species to break up into transverse segments, and in some cases to disintegrate as organizational entities, thereby releasing their constituent myxamoebae. The migrating pseudoplasmodium of *D. polycephalum*, like that of *D. discoideum*, shows a definite anterior end and the movement of the whole mass is apparently guided by it, although such movements appear to be at random rather than highly directed as in *D. discoideum*. Structurally, the pseudoplasmodium of *D. polycephalum* is composed of relatively few cells in transverse section because of its limited diameter, and throughout the greater portion of the body the constituent myxamoebae seem to have little distinctive form or

orientation. There is evidence that some of the peripheral cells are elongated in the direction of movement, and it is thought that the areas of the pseudoplasmodium where these occur are at the moment probably progressing more rapidly than other areas or, for that matter, the body as a whole. The myxamoebae in the extreme anterior region are characteristically oriented in the direction opposite to that of pseudoplasmodial movement. This, it is believed, is comparable to the situation in *D. discoideum*, but the area of transverse cellular orientation is much less extensive (see Bonner, 1944). Such directed action as the migrating body of *D. polycephalum* displays is believed to stem from this area.

It is in the process of sorocarp formation, or culmination, that *Dictyostelium polycephalum* exhibits its greatest difference from other members of the genus. It is noteworthy that the migrating pseudoplasmodium characteristically subdivides into a number of sorogens when it comes to rest preparatory to fructification; it is remarkable that these sorogens once formed remain sufficiently interdependent to culminate at a uniform rate; it is most extraordinary that the sorophores which are constructed individually by these sorogens should be formed contiguous to one another and that they should be cemented together to form a compound and, therefore, more rigid support for the elevation of the spore-bearing sori.

Lyophilized cultures of *Dictyostelium polycephalum* growing in association with *Dematium nigrum* and *Aerobacter aerogenes* have been deposited in the American Type Culture Collection, Washington, D.C., and in the Centraalbureau voor Schimmelcultures, Baarn, Holland.

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EXPLANATION OF PLATES

PLATE 1

- Fig. 1. *Dictyostelium polycephalum* growing on glucose (0.5 %) + peptone (0.1 %) agar medium in association with *Aerobacter aerogenes* and *Dematium nigrum*; incubation 3 weeks at 24–26°. $\times 2$.
- Fig. 2. Sorocarps and newly formed migrating pseudoplasmodia on an agar block (from fig. 1) viewed from the side. $\times 40$.

PLATE 2

Pseudoplasmodium formation in *Dictyostelium polycephalum* growing in two-membered culture with *Escherichia coli* on agar containing lactose (0.5 %) + yeast extract (0.05 %).

- Fig. 3. Early aggregating stage: centre of pseudoplasmodium appears as a dark ring, whereas the extent of aggregation is indicated by the broad halo surrounding this. $\times 10$.
- Fig. 4. A more advanced stage showing well-formed inflowing streams of myxamoebae. $\times 10$.
- Fig. 5. Fully developed aggregating pseudoplasmodium with anastomosing streams of myxamoebae. $\times 10$.
- Fig. 6. Aggregating pseudoplasmodium developing at colony margin; concentric bands are believed to result from wave-like convergence of myxamoebae. $\times 20$.
- Fig. 7. Unoriented, free-living vegetative myxamoebae. $\times 300$.
- Fig. 8. Portion of a pseudoplasmodial stream (as in fig. 5) showing uniformly oriented myxamoebae flowing downward toward the aggregation centre which is outside the picture. $\times 300$.
- Fig. 9. Long, thin migrating pseudoplasmodia emerging from two centres of aggregation. $\times 10$.

PLATE 3

Migrating pseudoplasmodia of *Dictyostelium polycephalum*

Fig. 10. Migrating pseudoplasmodia moving away from the sites where they originated. $\times 15$.

Fig. 11. Two well-formed migrating pseudoplasmodia. $\times 25$.

Fig. 12. A migrating pseudoplasmodium moving through the aerial mycelium of *Absidia coerulea*; note that this is supported at only three points throughout its entire length. $\times 15$.

PLATE 4

Sorocarp formation in *Dictyostelium polycephalum*

Fig. 13. Early stage in the formation of a coremiform fructification. The migrating pseudoplasmodium has become subdivided into several, vertically oriented sorogens, each of which will build a sorocarp side view. $\times 75$.

Fig. 14. A more advanced stage in a different fructification. $\times 60$.

Fig. 15. The coremiform fructification, consisting of 7 sorocarps, developed from the cluster of sorogens shown in fig. 13, photographed one day later. $\times 75$.

Fig. 16. Mature fructifications showing their general form and habit. $\times 40$.

PLATE 5

Coremiform fructifications of *Dictyostelium polycephalum* stained with rose bengal in 5 % aqueous phenol and photographed *in situ*

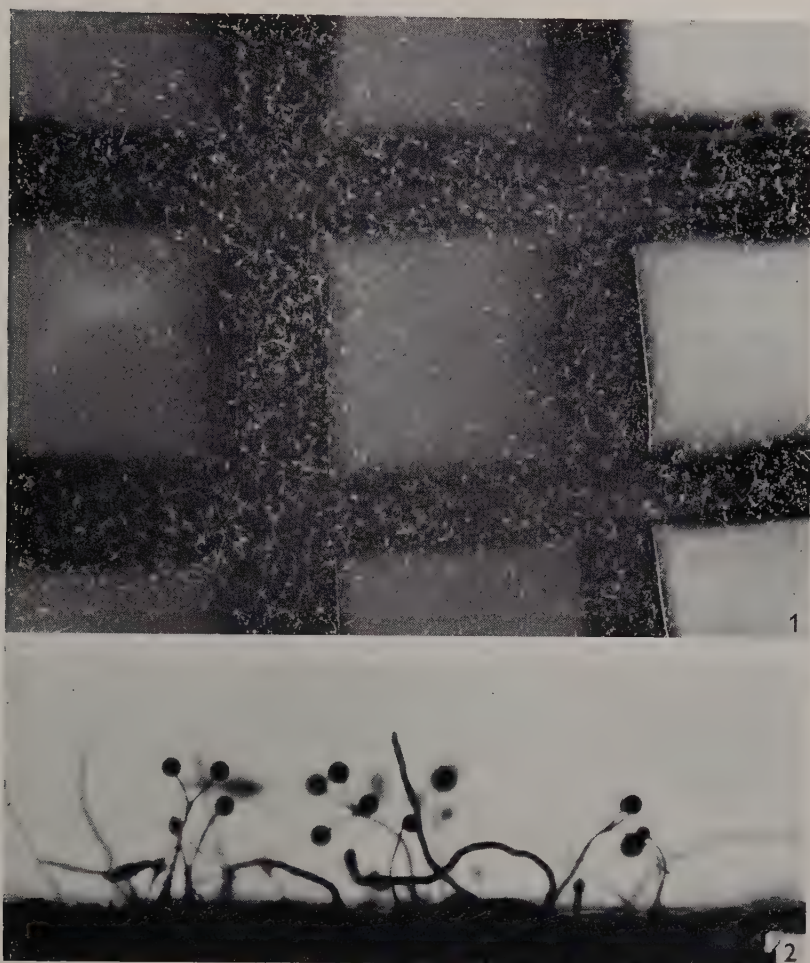
Fig. 17. A single sorocarp with its single globose sorus. $\times 50$.

Fig. 18. Coremiform fruiting structure composed of 3 sorocarps. $\times 50$.

Fig. 19. Compound structure composed of 5 sorocarps. $\times 150$.

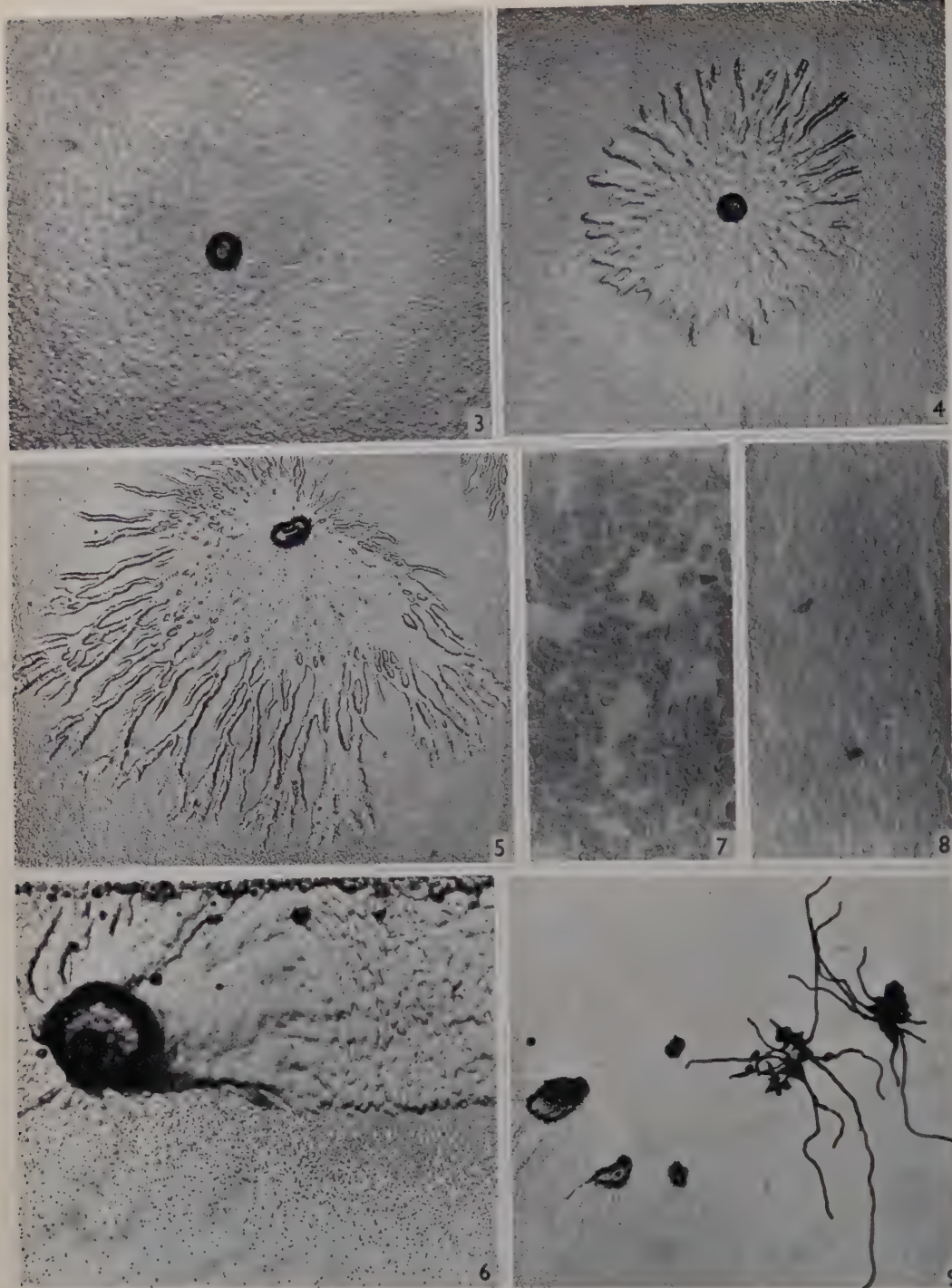
Fig. 20. Coremiform fructification showing cellular detail in its three adherent sorophores. The basal apron of slime anchors the structure to the substratum and holds it in an upright position. $\times 275$.

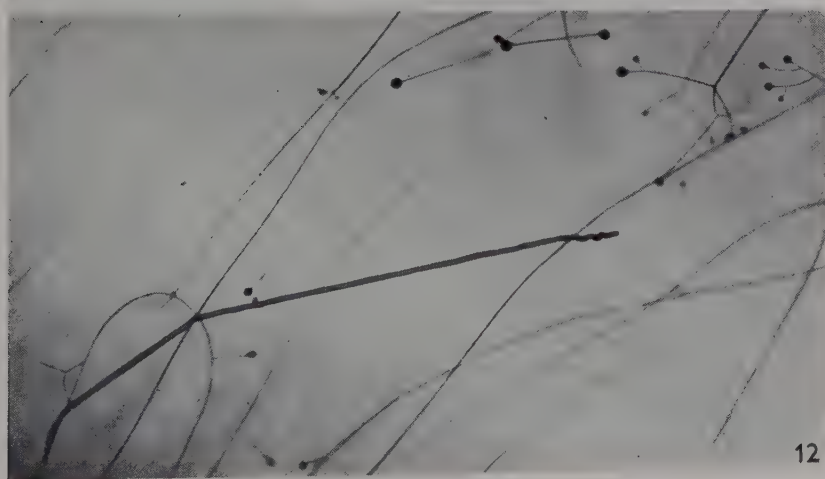
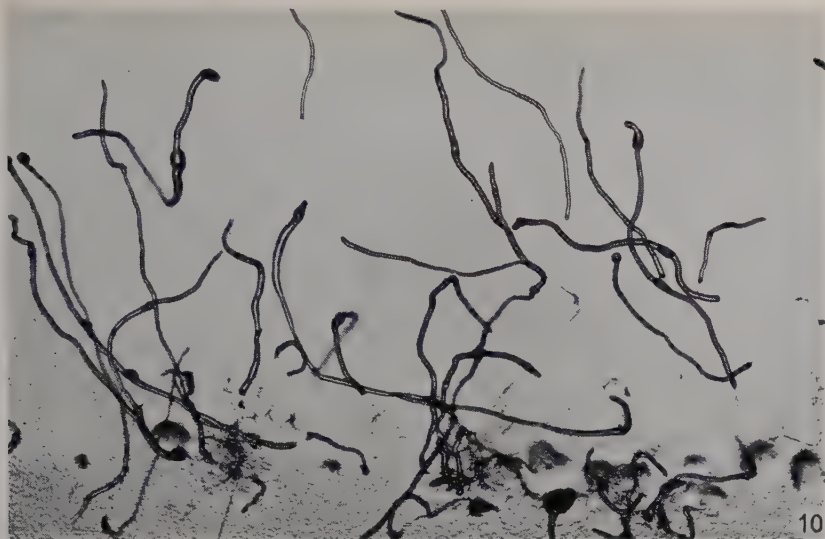
(Received 28 December 1955)



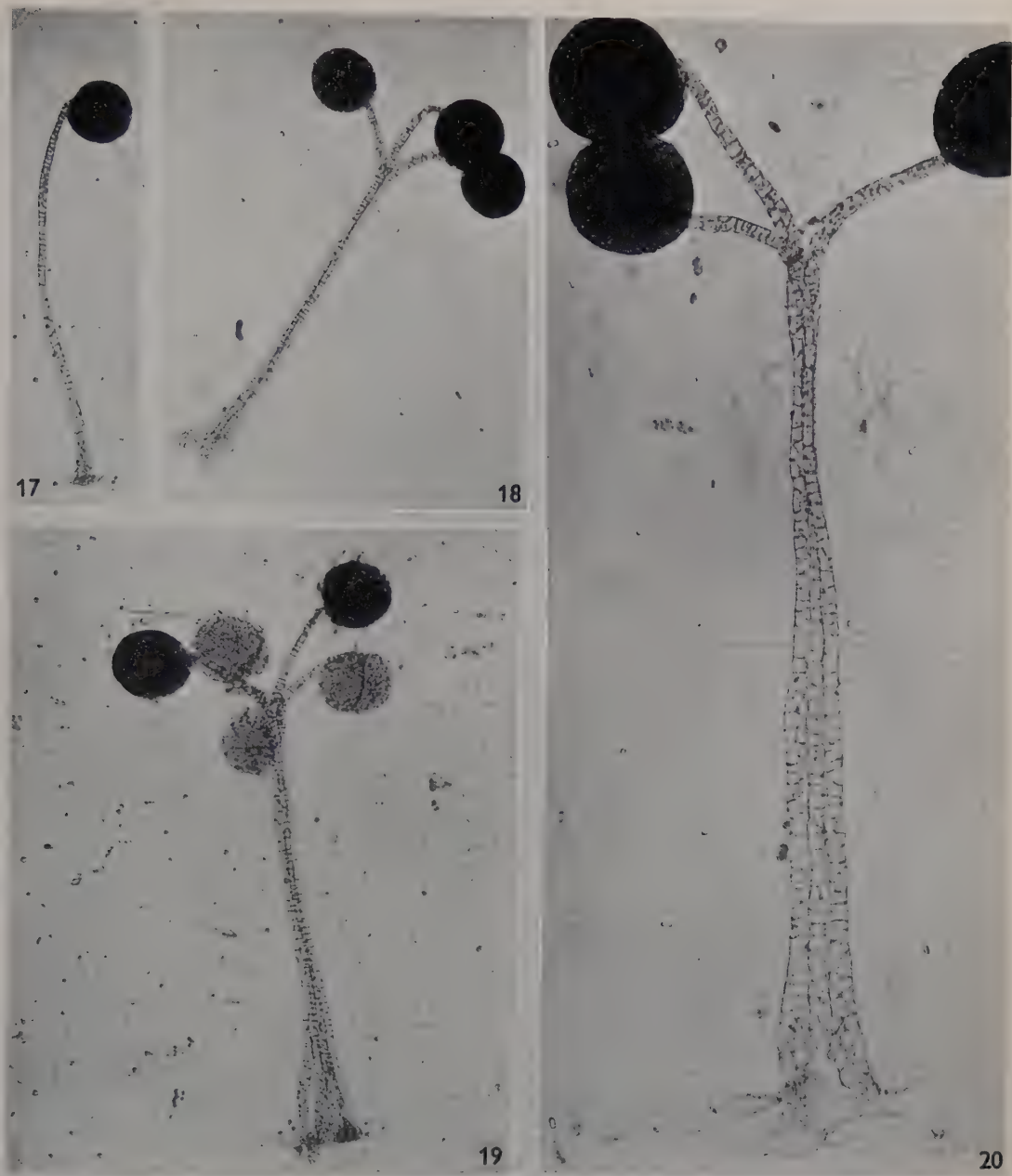
K. B. RAPER—*DICTYOSTELIUM POLYCEPHALUM* SP. NOV. PLATE 1

(Facing p. 732)









Criteria for Establishing the Validity of *in vitro* Studies with Rumen Micro-organisms in so-called Artificial Rumen Systems

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SUMMARY: Several criteria of normal rumen function which can be applied to *in vitro* studies with the whole rumen microbial population are suggested. These include: the maintenance of numbers and normal appearance of the bacteria, selenomonads and protozoa of the rumen; the maintenance of normal rates of digestion of cellulose, starch and protein, and of normal interactions between these; the ability to predict quantitative results *in vivo*. An 'artificial rumen' was constructed, consisting of a cellophan sac containing rumen liquor and substrate dialysing against a complex mineral solution whose composition was based on that found in rumen liquor, the whole being incubated at 39° in an atmosphere of nitrogen and carbon dioxide. This system was shown to meet the criteria which are suggested, with reasonable success for periods of about 8 hr.; over longer periods an increasing failure to meet the biological criteria was seen. For the microbial population to remain normal in numbers and activity it was shown to be necessary to use as test substrate *in vitro* only substances similar to the diet fed to the animal from which the rumen liquor inoculum was taken.

Of recent years there has been an increasing interest in the biochemistry and microbiology of the rumen. Because of the complexity of the rumen environment, many *in vitro* techniques have been applied including the use of: pure cultures of bacteria isolated from the rumen; washed suspensions of the whole or part of the rumen microbial population; cell-free enzyme preparations; whole rumen liquor under conditions which permit or encourage microbial multiplication. Under these last conditions, sometimes known as the 'artificial rumen' technique, it is essential to ensure that the microbial multiplication which takes place should proceed in a fashion similar to that found in the rumen of the living animal. There is normally present in the rumen a very large number of microbial species of widely differing physiological needs and capabilities, some found apparently only in the rumen and some found widespread in foodstuffs, soil, etc.; some of these microbial species may be expected to be able to multiply in almost any environment, and to metabolize almost any substrate that is likely to be tested. When, however, the environment or substrate is not that found in the rumen of the animal from which the rumen liquor inoculum was taken, then, in time, the results of this multiplication and metabolism may bear little or no relation to events in the rumen *in vivo*, since the organisms which multiply may be simply those most suited to the experimental conditions, and may well be only chance contaminants originally present in the rumen

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in quite small numbers. Since incubation periods of several days have frequently been used, the danger of this happening is very real.

Four main types of artificial rumen systems have been described: (1) undiluted or only slightly diluted rumen liquor is incubated with substrate in an all-glass impermeable system as used principally by Pearson & Smith (1943), Quin (1943) or Gray, Pilgrim & Weller (1951) and their respective co-workers; (2) whole rumen liquor is diluted to about half strength with a mineral solution resembling ruminant saliva in composition, and incubated with substrate in an impermeable system, as first described by Burroughs, Frank, Gerlaugh & Bethke (1950) but since used, with variations in detail, by many other workers; (3) various fractions of rumen liquor are used in an impermeable system, such as rumen liquor freed from protozoa by centrifuging (McNaught, 1951) or a suspension of all the rumen micro-organisms in a mineral solution (Marston, 1948); (4) rumen liquor, usually whole and undiluted, with substrate in a semi-permeable container, is dialysed against a mineral solution, as described by Louw, Williams & Maynard (1949), Huhtanen & Gall (1952) and Wasserman, Duncan, Churchill & Huffman (1952). Despite extensive use of some of these systems, not much has been done to establish the validity of the results in terms of normal rumen function, and some of what has been done is open to criticism.

Pearson & Smith (1943), on the basis of findings by the late F. Baker, who used formolized samples, claimed that their system remained normal for 2-4 hr. and that numbers and types of, mainly, the iodophilic bacteria showed no significant changes during that time; with the same technique, McNaught & Owen (1949) showed that that concentration of *o*-phenanthroline which suppressed urea utilization *in vitro* corresponded to a concentration of ferrous ion of the same order as that found *in vivo*. With a similar system with rumen liquor freed from protozoa McNaught (1951) found that the CO_2/CH_4 ratio in the evolved gases had an average value similar to that found *in vivo*, though the range of values was larger. Quin (1943) and McAnally (1943) showed that the rate of gas output following addition of glucose was similar *in vivo* and *in vitro*, but the incubation periods observed were very short.

Gray *et al.* (1951) successfully applied a number of criteria of normal rumen function to their system: the microscopically observed activity of the protozoa; the ratio of methane produced to fodder supplied; the digestibility of cellulose and pentosans; these were all similar *in vitro* and *in vivo*, but the authors noted that the rates of digestion of these substrates and of methane production were all only half those found *in vivo*. It is perhaps unfortunate that they used as buffer ammonium carbonate equivalent to 103 mg. N/100 ml., a concentration of ammonia very rarely found *in vivo* in the rumens of healthy animals even when fed a diet rich in a readily attacked protein (Gray & Pilgrim, 1952).

Burroughs, Frank, Gerlaugh & Bethke (1950) and Burroughs, Headley, Bethke & Gerlaugh (1950) in their system with successive fermentation periods each started by a 50% (v/v) inoculum of material from the preceding fermentation, claimed that there were no marked changes in numbers, size or predominant types of bacteria throughout, but that some of the types of

protozoa failed to survive. However, the main criterion of normal rumen function relied on by these workers was the high degree of cellulose digestion obtained; but two points must be noted. First, the absolute amount of cellulose used was small, about 3 g./l. artificial rumen contents in early experiments, later 10 g./l., or occasionally up to 20 g./l., though animals fed high roughage diets, such as were used to supply the inoculum for these experiments, would receive perhaps 50 g. cellulose/l. rumen contents/day, as well as hemicelluloses, etc., which might well involve the same digestive enzymes, so that the actual amount, in g. cellulose/l., digested/day in the artificial rumen was much less than was digested *in vivo*. Secondly, the criterion relied on, namely the digestibility of cellulose over several fermentation periods, does not seem very reproducible. In two successive papers where detailed figures are given (Arias, Burroughs, Gerlaugh & Bethke, 1951; Burroughs, Latona, De Paul, Gerlaugh & Bethke, 1951), when examination is made of the results from the control flasks containing 9 g. cellulose/900 ml. diluted rumen liquor only, and followed through four successive fermentations (there are five such in the first paper and six in the second), a statistically significant increase in cellulose digestibility can be seen during the second fermentation period, and a significant fall in the third period, while in the fourth period there was a highly significant but unexplained difference between the results reported in the first paper (where there was a marked drop in digestibility), and those in the second paper (where there was little if any decrease).

Using a somewhat similar system, though with only one fermentation period of about 40 hr., Brooks, Garner, Gehrke, Muhrer & Pfander (1954) claimed that the numbers of bacteria before and after incubation were approximately equal, and that the effects of added fat on cellulose digestion *in vivo* and *in vitro* were very similar. However, examination of the figures shows that a dose of 32 g. corn oil/sheep decreased cellulose digestibility from 41.9 to 20.0 %, while a dose of 160 mg. corn oil/25 ml. diluted rumen liquor *in vitro* (the same concentration, assuming a rumen volume of 5000 ml.) decreased cellulose digestibility from 36.2 to 2.2 %, a much more marked effect. Brooks, Garner, Muhrer & Pfander (1954) also tested the effects of various steroids on cellulose digestibility *in vivo* and *in vitro*, and again a more marked effect was obtained *in vitro*.

Louw *et al.* (1949) showed that the semipermeable artificial rumen permitted better digestion of cellulose than an impermeable system with the same inoculum. Gall & Glaws (1951) and Huhtanen, Saunders & Gall (1954) showed that the bacteria seen in a Gram-stained film or grown in anaerobic culture were more nearly similar at the end and the beginning of incubation, and the protozoa were more motile throughout, with a semipermeable system as compared with an impermeable system.

In summary, then, both biological and chemical criteria have been used in attempts to establish the validity of the results obtained with these artificial rumen systems. Biologically, attention has been paid to: motility of the micro-organisms, particularly the protozoa; predominant morphological or cultural types of bacteria; in a few cases, numbers of bacteria, though no detailed

figures have been published. Chemically, most attention has been paid to the proportion of cellulose digested, though other relative values have been used; little attention has been given to the actual rates of digestion of substrate. It should be noted that in no case where incubation has been continued for more than a very few hours has complete success been reached in meeting even the few criteria that most authors have been content to adopt. It is the intention of this paper to discuss a number of criteria of normal rumen function and to apply these to an artificial rumen system based on that of Louw *et al.* (1949).

METHODS

Animals. Cheviot ewes or wethers were fitted with rumen fistulas by Dr A. T. Phillipson. Except where otherwise mentioned, the diet was: 300 g. hay, 300 g. groundnut meal and 300 g. flaked maize/day, fed in two portions at 07.30 and 19.30 hr. Samples of rumen liquor were removed at 07.00 hr. strained through eight thicknesses of surgical gauze and used without delay.

Materials. Groundnut meal, herring meal, Paisley meal and casein were as described by Annison, Chalmers, Marshall & Syngé (1954). Starch used in *in vitro* tests was Soluble Starch (British Drug Houses, Ltd.); potato starch was used in feeding experiments. Cellulose was filter-paper ground in a hammer mill. All meals, etc., used in *in vitro* work were finely powdered in a hammer mill before use.

Chemical estimations. Ammonia-N was estimated by adding 1.0 ml. rumen liquor or other fluid to 1.0 ml. 0.1 N-HCl, diluting to 5.0 ml. and filtering (McDonald, 1952), liberating the NH_3 in the filtrate with K_2CO_3 according to Conway & O'Malley (1942) and absorbing it in the boric acid indicator solution diluted with water to half strength (E. F. Annison & J. C. Wood, private communication). This diluted indicator solution gives a much more sensitive end-point for amounts of ammonia up to about 0.05 mg. $\text{NH}_3\text{-N}$, and will quantitatively absorb up to about 0.16 mg. $\text{NH}_3\text{-N}$. Volatile fatty acid was estimated by steam distillation of 2.0 ml. of the HCl filtrate obtained above with 1.0 ml. syrupy H_3PO_4 in the apparatus of Markham (1942); 50–60 ml. of distillate were collected, aerated with CO_2 -free air for at least 3 min. and titrated with 0.01 N-NaOH (CO_2 -free). An approximate estimate of starch in rumen liquor was made according to a method of Dr P. N. Hobson (private communication). Rumen liquor (2.0 ml.) was placed with 0.6 ml. 5 N-NaOH in a 10 ml. measuring flask, heated in a boiling water-bath for 20 min., cooled and diluted to the mark with water. After standing for a few minutes, the mixture was centrifuged for 20 min. at 2500 r.p.m. and 1.0 ml. of the supernatant fluid added to c. 60 ml. water; 4 drops 5 N- H_2SO_4 and 1.0 ml. Spekker iodine (0.2 % I_2 in 2.0 % KI, w/v) were added, the whole made up to 100 ml. and the colour measured immediately in an EEL portable photoelectric colorimeter, using the red filter. Experiment showed that each scale division (0.01 optical density unit) was approximately equivalent to 0.1 g. starch/100 ml. rumen liquor.

Microbial counts. Strained rumen liquor was diluted 1/5 with 10 % (v/v)

formalin (Baker, 1943), and after shaking, the protozoa were counted in a Manners counting chamber (0.2 mm. deep) and the bacteria in a Helber counting chamber (0.02 mm. deep) (counting chambers supplied by Hawksley & Sons Ltd., London). Phase-contrast microscopy was used throughout. By counting about 2000 organisms of each of the numerically more important types of micro-organism, replicate counts were made to agree within $\pm 5\%$. Special precautions were needed with the Helber counting chamber, since its depth was less than at least two dimensions of the larger protozoa; the actual depth of fluid in the chamber was tested each time by means of the calibrated fine-focusing mechanism of the microscope. Any preparation which showed a burst holotrich protozoon on low-power examination was rejected, as the grains of protozoal polysaccharide and other intracellular particles liberated from such a burst organism were at times difficult to distinguish microscopically from bacteria.

Artificial rumen apparatus. The apparatus (Fig. 1) consisted of a piece of glass tubing, 20×5 cm. closed with rubber bungs at each end and containing a cellophan dialysing sac, 12×3 cm., also closed with rubber bungs. This sac contained usually 50 ml. rumen liquor and the substrate, previously moistened with the mineral solution described below. The outer chamber was filled with the mineral dialysing solution to the same level as the rumen liquor, the volume being noted. A slow stream of nitrogen containing 5% carbon dioxide (British Oxygen Co. Ltd.) was bubbled through both solutions, partly to avoid loss of material through frothing, which was troublesome with some substrates, any material carried over in froth being measured in the ordinary analysis of the mineral solution. The pH values of the solutions in both compartments were adjusted to between 6 and 7 when necessary, using $m\text{-H}_3\text{PO}_4$ or a mixture of $0.5\text{ M-K}_2\text{HPO}_4 + 0.5\text{ M-Na}_2\text{CO}_3$; when the substrate approximated the composition of a normal diet, little adjustment was needed, but when single substrates, particularly starch, were used, considerable alterations of pH value occurred. The whole apparatus was immersed in a water-bath at 39° .

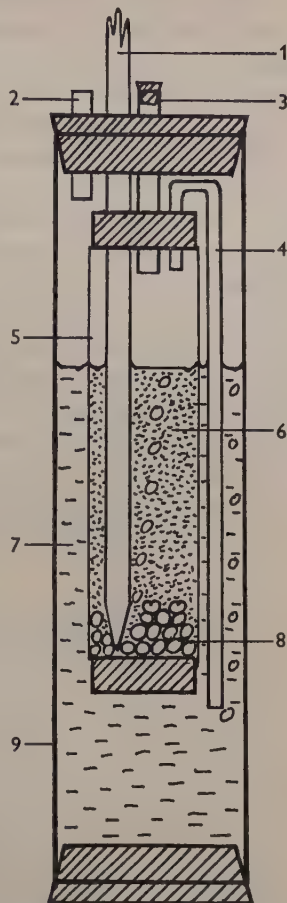


Fig. 1. Artificial rumen apparatus (about $\frac{1}{2}$ scale). 1, Inlet for gas mixture; 2, access to mineral solution and outlet for gases; 3, access to inner sac; 4, duct for gases; 5, cellophan dialysing membrane; 6, rumen liquor and substrate; 7, complex mineral solution and products of diffusion from rumen liquor; 8, glass beads to facilitate mixing by rotating the whole apparatus by hand; 9, outer glass container.

At the conclusion of the experiment, the volumes of fluid in both compartments were measured, and the volumes at intermediate times were calculated, allowing for known volumes removed as samples and assuming that any flow due to osmotic differences, etc., was constant. The ratios of the outer to the inner volumes were then calculated; in the apparatus described, these ratios were always between 2.9 and 4.1, usually about 3.5. The total 'concentration' of any metabolite was then calculated as the concentration inside the dialysing sac + the product of the concentration in the outside compartment (less the value at zero time) and the above ratio; this gives in effect what would have been the concentration of the metabolite in the dialysing sac if none had diffused away. All concentrations given in this paper for diffusible substances are calculated in this way, so that, for example, the concentrations of ammonia actually in contact with the micro-organisms would be considerably less than the figures given, perhaps one-third, and consequently well within physiological limits at all times.

The influence of the mineral base used in work with rumen micro-organisms *in vitro* has been inadequately examined, it being customary to use either a simple buffer or a complex solution of composition similar to saliva. In the present work, a similar solution (solution I of Table 1), was used for all except

Table 1. *Composition of mineral dialysing solutions, compared with the composition of saliva and rumen liquor of sheep*

Element or ion	Concentration (mg./100 ml.)			
	Solution I	Solution II	Saliva*	Rumen liquor†
K	74	117	12-46	99-175
Na	242	197	352-462	137-202
NH ₄ -N	14	14	9-35	—
Ca	4	10	0.2-3.0	10-21
Mg	2.4	6	0.4-1.1	7-20
PO ₄ -P	155	101	19-129	28-81
SO ₄ -S	3.2	24	—	—
Cl	126	87	19-238	35-92
HCO ₃ ⁻	Saturated	Saturated	Saturated	Saturated
Fe	0.2	0.2	—	—
Mn	0.13	0.13	—	—
Zn	0.1	0.1	—	—
Co	0.05	0.05	—	—
Cu	0.05	0.05	—	—
Acetate ⁻	180	216	—	—

Solutions I and II also contained 2 ml./l. of the 10% cysteine solution of Huhtanen, Rogers & Gall (1950).

* See McDougall (1948).

† See Phillipson (1953).

the last two experiments described. On noting the differences between the composition of this mixture and that of rumen liquor (Phillipson, 1953), the mixture was amended accordingly, and the following differences in functioning of artificial rumens dialysing against these two solutions (solutions I and II of

Table 1) were noted: motility of the protozoa and the selenomonads* was better maintained with solution II; the rate of ammonia production in the absence of added substrate was slightly but significantly lower; the rate of ammonia production in the presence of added casein was slightly but significantly higher with solution II than with solution I. Solution II was therefore used in the remaining experiments reported here. Similar differences were noted when comparing solution II with a simple phosphate NaCl buffer. After completion of this work, the influence of various mixtures of volatile fatty acids on one of the more important rumen bacterial species was reported by Bryant & Doetsch (1954), and some slight improvements appeared to be made on replacing some of the chloride and acetate in solution II with propionate, isobutyrate, valerate and isovalerate in the artificial rumen system at concentrations within the limits described by Annison (1954). In assessing the importance of some of these substances in the dialysing system, it must be realized that, owing to the closed nature of the system, no substance could diffuse out of the dialysing sac to give a final concentration lower than about a quarter of its initial value.

CRITERIA OF VALIDITY OF THE ARTIFICIAL RUMEN USED

Numbers of micro-organisms

The numbers of micro-organisms before and after incubation cannot be used to estimate true mean generation times owing to the complexity of the system: it is probable, though not proven, that the protozoa consume bacteria in large numbers either to satisfy nutritional needs or simply as attached to food particles; protozoa can be seen to consume other protozoa; bacteria and possibly selenomonads can be seen to consume protozoa; the quantitative significance of these processes in the economy of the rumen is, however, quite unknown. Table 2 shows that in the presence, though not in the absence, of substrate the numbers of bacteria and protozoa were maintained approximately constant in the artificial rumen for 7 hr. The decrease in the number of selenomonads appears to be due to the nature of the substrate used since it was noted in several experiments with casein \pm starch as substrate, whereas in an experiment using starch + cellulose + purified groundnut meal protein there was a slight rise in numbers over 8 hr. Too much significance is not claimed for counts of this nature, except when using as substrate the diet of the animal from whose rumen the inoculum was taken and comparing changes in microbial numbers *in vivo* and *in vitro*; they do, however, seem to show that no noticeable change occurred in the balance of micro-organisms within the time stated.

Motility of micro-organisms

In rumen liquor examined immediately after removal from the animal, about 90 % of the protozoa and 80 % of the selenomonads appeared actively motile. After 8 hr. in the artificial rumen, the motility of the holotrich protozoa appeared unaffected; about 60 % of the entodinia were actively motile, about

* Term used here to include organisms 3, 4 and 5 of Moir & Masson (1952); see also Lessel & Breed (1954); Judicial Commission (1955).

25 % sluggishly motile (in the rumen contents of the animals used in this work other genera of oligotrich ciliate protozoa were either absent or present in too low numbers to be accurately counted). About 40–50 % of the selenomonads were actively motile. Owing to the difficulty of identifying species with certainty, motility in bacteria was not examined. The motility of the protozoa and selenomonads did not appear to depend to any great extent on the presence

Table 2. *Numbers of micro-organisms present in rumen liquor before and after 7 hr. incubation in the artificial rumen apparatus*

Organism	Counts (no./ml.)			
	No added substrate		1 % casein and 1.8 % starch added	
	Initial	Final	Initial	Final
Total protozoa $\times 10^{-6}$	1.92	1.64	1.78	1.84
<i>Entodinium nanellum</i> * group $\times 10^{-5}$	7.4	6.9	7.7	6.7
<i>Entodinium longinucleatum</i> * group $\times 10^{-5}$	2.9	2.8	3.2	3.3
<i>Entodinium caudatum</i> * group $\times 10^{-5}$	5.0	4.1	4.6	4.9
Total selenomonads $\times 10^{-8}$	3.9	3.0	3.2	2.4
Total bacteria $\times 10^{-10}$	3.5	2.7	3.4	3.6
Chains of large streptococci† $\times 10^{-7}$	1.9	1.5	2.0	2.4

* Species differentiation by phase-contrast examination alone is almost certainly unsatisfactory, but many of the protozoa seen can be placed in groups resembling species as described by Bhatia (1936). Other, unidentifiable, *Entodinia* spp. were also seen, and the total number of entodinas constituted about 98 % of the total protozoal count in this instance.

† Organism 27 of Moir & Masson (1952).

or absence of substrate, at least within the period studied, possibly owing to the removal of dead organisms by living ones. Motility appears to be a sensitive criterion of normal rumen function, but care must be exercised to ensure maintenance of temperature and anaerobiosis of the specimen during examination.

Proportion of dividing protozoa

It was thought that the proportion of organisms showing signs of being about to divide might be a sensitive measure of the viability of the protozoa. Unfortunately, this proportion fluctuates *in vivo* for causes so far not determined. In specimens of rumen liquor, taken at various times from one sheep on one diet, the proportion of entodinas dividing was between 1.2 and 1.9 % on seven occasions, between 0.6 and 1.2 % on five occasions and just over 3 % once; these fluctuations did not appear to depend to any great extent on the time after feeding at which the sample was taken. Nevertheless, it is probably significant that in all experiments where this proportion has been estimated, it has decreased to about half its initial value after 8 hr. in the artificial rumen. The dividing organisms present at the end of the experiment could not have been those seen at the beginning: if dividing entodinas were followed about a slide kept at about 39° under the microscope, division was seen to be completed in 15–25 min. It may be noted that a median value of 1.5 % organisms

dividing and taking 20 min. to complete division indicates a mean generation time of 22 hr., about the value found by Hungate (1942) from his work with cultures.

Effect of long incubation periods

Over a period of nearly 4 days, on some five occasions each day, about 15–20 % of the contents of the dialysing sac were removed and replaced with mineral solution; 3 times/day the contents of the outer compartment were completely renewed; twice a day a mixture equivalent to the animal's feed was added to the sac; except for a period of some 11 hr. each night when no attention was given, the system was mixed and neutralized at intervals. The experiment was terminated when the cellophan dialysing sac split along the line of folding. Throughout the experiment, the protozoa, selenomonads and spirilla were actively motile, except in the first specimens examined in the morning, when motility was sluggish. All morphological types of micro-organism present at the start of the experiment were present at the finish, though the proportion of spirilla and of the large streptococci increased; the latter organism has not yet been cultivated outside the rumen, suggesting that it has some special nutritional needs supplied presumably by commensal growth of other micro-organisms, so that an increase in numbers in this experiment would not suggest a gross abnormality in the medium. The mechanical dilution was such that the final concentration was 0.066 of the initial; the final number of entodinas was 0.24 of the initial, so that there was a net multiplication of 3.6 times. That is, despite the long period during the night when no attention was given to the apparatus, the microbial population was still reasonably typical of the rumen over 4 days. Long-term experiments not involving progressive dilution, etc., that is where rumen liquor was incubated with substrate for 24–48 hr., without any other additions or removals, showed a much more rapid deterioration of the microbial population, the protozoa and several morphologically distinctive bacterial species, particularly *Oscillospira guilliermondii* or organism 1, and organism 25 of Moir & Masson (1952), markedly decreasing in numbers within 24 hr.

Cellulose digestion

Although cellulose digestion is one of the most frequently used criteria of normal functioning of artificial rumen systems, there are certain disadvantages in laying stress on this, even though it is one of the most characteristic functions of the rumen. There is, first, considerable doubt as to the actual rate of cellulolysis in the rumen as distinct from an overall rate for the whole animal. The rate-limiting step in cellulose digestion is unknown; if it should occur at a very early stage, where the products would still be measured as cellulose by most techniques in common use, it might well be that the rate of digestion of the cellulosic contents of the rumen as ordinarily measured (and roughage on the average probably remains in the rumen 24 hr. or more), might be very different from the rate of digestion of cellulose newly introduced into an artificial system. Moreover, since it is practically essential to strain the rumen

liquor for use *in vitro*, some diminution of the rate of digestion of cellulose is to be expected, since many of the cellulolytic micro-organisms would remain attached to the large plant particles removed in straining. The sheep used to provide the inoculum in the present experiments was fed a high concentrate diet, containing probably less than 200 g. cellulose/day; since the diet was of a type found by Head (1953) to depress cellulose digestibility, probably considerably less than 100 g. cellulose was being digested each day. Addition of 2.0 g. cellulose to 50 ml. rumen liquor in the artificial rumen apparatus resulted in the production of 1.1 mequiv., probably 80 or more mg. volatile fatty acid in 8 hr.; 1 g. volatile fatty acid is produced from about 2 g. cellulose (Carroll & Hungate, 1954), so that the rate of cellulose digestion in this apparatus was about 10 g./l. rumen liquor/day, or about 50 g./day for the animal. Considering the various assumptions made, this agreement seems fairly good. This *in vitro* rate of cellulose digestion is exceeded only by the figure of 18 g./l./day given by Louw *et al.* (1949).

Starch digestion

The rate of starch digestion *in vitro* as measured by the rate of disappearance of starch appears to depend considerably on the diet of the animal from which the rumen liquor inoculum was taken, as shown in Table 3. Dr P. N. Hobson (private communication) found in the case of sheep no. 1054 that few starch grains were visible microscopically in the rumen contents 12 hr. after feeding 100 g. potato starch, a rate of starch digestion probably equivalent to the 2.0 g./l. rumen contents/hr. found *in vitro*.

Table 3. *Effect of diet of animal on rate of starch digestion in vitro*

Animal	Daily diet	Rate of starch digestion, g./l. rumen contents/hr.
969	900 g. hay	0.5
43	300 g. hay, 300 g. flaked maize, 300 g. groundnut meal	1.0
377	600 g. hay, 300 g. concentrates	1.5
70	600 g. hay, 300 g. flaked maize	1.0
416	600 g. hay, 300 g. maize gluten	1.3
1054	300 g. hay, 200 g. potato starch, 500 g. concentrates	2.0

Casein digestion

Dr I. W. McDonald (private communication) found that when 100 g. casein was fed to a sheep, the last traces of bound phosphate, presumed to come from the casein, passed through the abomasum about 8–10 hr. after feeding. The peak in the curves for ammonia production in the rumen found by Chalmers and colleagues (Annison *et al.* 1954; Chalmers & Synge, 1954; Chalmers, Cuthbertson & Synge, 1954) usually occurred about 4–5 hr. after feeding 50–100 g. casein. It seems reasonable to suppose that the time for complete digestion in the rumen is intermediate between these values, say 6–8 hr. In the artificial rumen, the curve for ammonia production from added casein first rises at a rate independent of the amount added, and then, at a time which depends on

that amount, flattens out to a rate of ammonia production equal to that found in the absence of added substrate (see Fig. 2). It seems probable that this point of inflexion corresponds to the completion of digestion of the casein. In a considerable number of experiments ammonia production due to 0.7 g. casein in 50 ml. rumen liquor ceased after 5–8 hr., corresponding to an amount of 70 g. casein/sheep digested in a similar time, or a rate of casein digestion of about 0.3 g. N/l./hr.

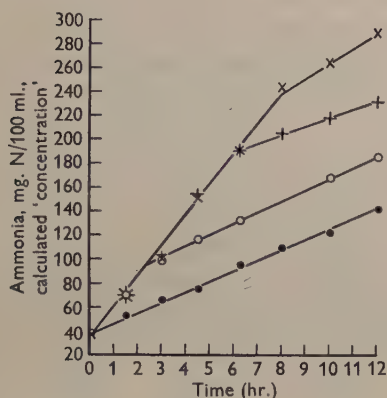


Fig. 2

Fig. 2. Ammonia production from varying amounts of casein in the artificial rumen apparatus. ●, no added substrate; ○, 0.3 g. casein/artificial rumen (70 mg. N/100 ml.); +, 0.7 g. casein/artificial rumen (180 mg. N/100 ml.); ×, 1.2 g. casein/artificial rumen (320 mg. N/100 ml.).

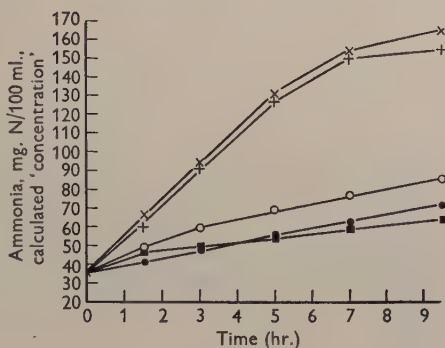


Fig. 3

Fig. 3. Rates of production of ammonia from various protein foodstuffs in the artificial rumen. ●, no added substrate; ■, 2.2 g. paisley meal/artificial rumen (184 mg. N/100 ml.); ○, 0.8 g. herring meal/artificial rumen (178 mg. N/100 ml.); +, 1.1 g. groundnut meal/artificial rumen (180 mg. N/100 ml.); ×, 0.7 g. casein/artificial rumen (184 mg. N/100 ml.).

Ammonia production from other proteins

The relative amounts of ammonia produced in the artificial rumen from groundnut meal, herring meal, Paisley meal and casein (Fig. 3) appear to correspond to the relative amounts found to be produced *in vivo* by Chalmers and colleagues, namely, slightly less from groundnut meal than from casein, considerably less from herring meal, and little, if any, from Paisley meal.

Interaction between starch and casein

It has been known for some time that the addition of starch lowers the ammonia concentration in the rumen, whether the ammonia is largely produced from urea (Mills, Booth, Bohstedt & Hart, 1942) or from protein (McDonald, 1952; Annison *et al.* 1954). This was also found in the artificial rumen (Table 4), where the addition of starch lowered endogenous ammonia production nearly as much as total ammonia production from casein.

The end products of digestion

The nature of relative quantities of the end products of digestion have been used by various workers to demonstrate normality of function of an *in vitro* system. In the present work, it was found that ammonia and volatile fatty acids were produced in roughly equimolar quantities (Table 4). In other experiments, the output of ammonia-N, in m-equiv./100 ml., from

Table 4. *Digestion of starch and casein, separately or together, in the artificial rumen, in 9 hr.*

	Contents of artificial rumen apparatus: 50 ml. rumen liquor with			
	No added substrate	1.8 % (w/v) starch	1.0 % (w/v) casein	1.8 % (w/v) starch and 1.0 % (w/v) casein
Starch (g./100 ml.)				
Initial	0.20	1.83	0.20	2.00
Final	0.00	0.70	0.03	0.60
Amount digested — blank	—	0.93	—	1.23
Volatile fatty acid (m-equiv./100 ml.)				
Initial	5.9	6.0	6.0	6.1
Final	16.5	25.0	24.7	32.8
Amount produced — blank	—	8.4	8.1	16.1
Ammonia-N (mg./100 ml.)				
Initial	22.8	22.8	22.8	22.8
Final	57.8	37.5	140.3	116.2
Amount produced — blank	—	-20.3	+82.5	+58.4

12.5 m-equiv. N casein/100 ml., was 5.9, 6.4, 5.8, 5.7 and 5.9; the output of volatile fatty acid in m-equiv./100 ml. in the same experiments was respectively 8.1, 5.3, 7.9, 6.7 and 5.0. These figures correspond to the roughly equimolar amounts of ammonia and volatile fatty acids found in the rumen after feeding several diets by el-Shazly (1952) and Annison (1954). It was also found that considerable amounts of volatile fatty acid were produced from starch, concentrates, hay and cellulose in the artificial rumen.

Quantitative prediction of in vivo results

Two rations, E and H, were composed such that they gave equal ammonia production in the artificial rumen, when rumen liquor from sheep 70 fed the standard groundnut meal diet was used as inoculum. The composition of these rations is given in Table 5; they were fed to two sheep, 377 and 416, in turn. Three weeks after beginning the diet, the rumen ammonia production *in vivo* was determined, following the methods of Chalmers *et al.* (1954). The results (Figs. 4, 5) showed that reasonable prediction had been obtained, allowing for some variation between the two animals. At the same time, a third diet, G, was tested; this also had given similar ammonia production in the preliminary *in vitro* test, but *in vivo*, markedly less ammonia was produced from diet G than from either of diets E or H. This was thought to be because the lower nitrogen

content of the diet supported a less numerous, and hence less active, microbial population in the rumen. This is an example of the dangers of relying on *in vitro* tests to predict behaviour *in vivo*, where the substrate tested is very different from the diet of the animal used to supply the rumen liquor inoculum.

Table 5. Daily rations fed to sheep

Constituent	Quantities fed (g./day)			
	Diet E	Diet H	Diet G	Standard*
Hay	600	600	600	300
Flaked maize	—	—	—	300
Concentrates 411†	150	150	150	—
Groundnut meal E‡	100	—	—	—
Herring meal	—	130	—	—
Groundnut meal	—	—	30	300
Total N content	19	23	12	30

In diets E, H and G the hay was fed in two lots at 11.00 and 16.00 hr., the remainder at 07.00 hr.; half the standard ration was fed at 07.30 hr., half at 19.30 hr.

* Diet fed to sheep used to supply rumen liquor inoculum for preliminary *in vitro* tests.

† 4 parts ground maize, 1 part wheat bran, 1 part crushed oats.

‡ A special groundnut meal, solvent-extracted under harsh conditions and of low salt peptizability, supplied by the Research Department of J. Bibby and Sons Ltd., Liverpool.

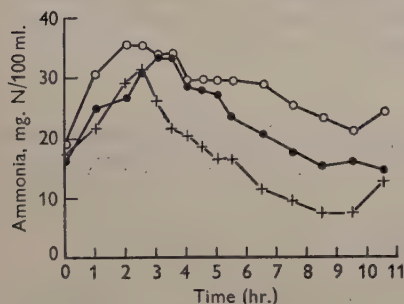


Fig. 4

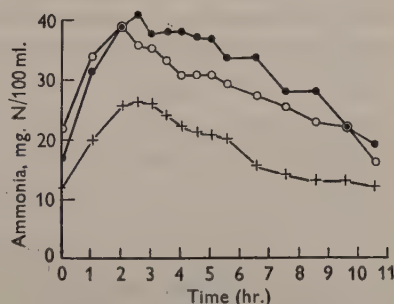


Fig. 5

Fig. 4. Ammonia production in the rumen of sheep 377 from the three rations; ●, E; ○, H; +, G.

Fig. 5. Ammonia production in the rumen of sheep 416 from the three rations; ●, E; ○, H; +, G.

DISCUSSION

For convenience in the calculations above it was assumed that the volumes of the rumen contents of the sheep used were of the order of 5 l. This is probably an underestimate for the larger animals, but minor discrepancies would not affect the argument.

In the present state of knowledge of the biochemical and microbiological events in the rumen, there seem to be no grounds for relying on one or a few criteria of normal rumen function; rather as large a number and variety of criteria as possible should be used. While biological criteria are probably simpler to apply, they have more value in long-term investigations than in work lasting no more than a few hours. On the other hand, the chemical

criteria such as the rates of digestion of substrate or of production of metabolite can be quite sensitive over short periods of time. In this connexion, the expression of results in units such as g./l./hr. or day, rather than as digestibilities or other relative values, is to be recommended as making comparisons between different techniques simpler.

The latter part of the last experiment shows that not only must the physical environment in an artificial rumen approximate to normal (maintenance of a suitable temperature, pH value, gas phase, provision for removal of metabolites) but also the substrates tested must approximate in nature and quantity to the diet of the animal from which the rumen liquor inoculum was taken, if the *in vitro* results are to serve as indications of results *in vivo*. In other cases the *in vitro* results may serve to show what might happen *in vivo* on first changing the ration of the animal, but it is well known that several days or weeks are needed for the rumen microbial population to adapt to a new diet, behaviour during the first few days following a change of diet being relatively unpredictable.

The too frequent practice of adding *in vitro* to rumen liquor taken from an animal on some standard diet, a single dose of some substance or mixture unrelated to the diet, incubating for lengthy periods of time and using the results to attempt to predict the effects of feeding that substance or mixture in addition to or even instead of the standard diet, with little or no attempt to show that the *in vitro* system used successfully reproduces the features of rumen function *in vivo*, is to be deprecated.

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Proteolysis by Rumen Micro-organisms

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SUMMARY: Toluene-treated washed suspensions of rumen bacteria break down proteins largely to amino acids; in the absence of toluene bacterial deaminases are active. Unlike the deaminases, the presence of proteases does not depend, to any great extent, on the presence of readily attacked protein in the diet of the host animal. Extracts of acetone-dried powders of the bacteria also show proteolytic activity. Rumen protozoa are also proteolytic, and ammonia appears to be the end product of their nitrogen metabolism. Ammonia production due to the protozoa is not as sensitive to toluene as is the case with bacteria. Much of the ammonia production in the rumen in the absence of substrate appears to be due to the endogenous metabolism of the protozoa. Extracts of acetone powders, and extracts prepared by simple freezing and thawing of the protozoa, contain active proteases.

In an artificial rumen apparatus it was shown that when digestion was complete, about half the N and C of added casein could be recovered as ammonia and volatile fatty acids respectively. Most of the remainder could not be accounted for analytically, and was presumed to be used for microbial growth, which had occurred. When starch or some other polysaccharides were added to the artificial rumen apparatus as well as casein, the production of ammonia was lowered. This was shown not to be due to any effect on proteolysis or deamination, and was presumed to be due to the increased utilization for microbial growth of some breakdown product of casein.

From at least the time of Zuntz (1891), it has been suspected that micro-organisms of the rumen split the proteins fed to the host animal; there has been, however, little unequivocal direct evidence for this proteolytic activity. Schlottke (1936) showed *in vitro* that glycerol extracts of rumen protozoa contained a protease active at pH c. 6.1, and also a dipeptidase, but was unable to find much activity in glycerol extracts of rumen bacteria. However, Sym (1938) showed active proteolysis at pH 6.3 by suspensions of rumen bacteria and of protozoa, and also by extracts of acetone powders of these micro-organisms; he found peptidase activity weak in his preparations. Both these authors found little or no free protease in the supernatant rumen liquor. Nikitin (1939) and Pearson & Smith (1943) demonstrated proteolysis by rumen micro-organisms *in vitro*. Indirect *in vivo* evidence that food protein is converted into microbial protein, with the presumption of intermediate proteolysis, was given by McDonald (1954) who reviewed the earlier evidence.

The concentration of free amino acids in the rumen is at all times low (Chalmers & Synge, 1954*b*), but breakdown products of the amino acids, ammonia (McDonald, 1948, 1952) and volatile fatty acids (el-Shazly, 1952*a*) are found in high concentration when the diet of the animal contains adequate and suitable protein. Both the prior treatment and the nature of the protein affect the amount of breakdown products found. Chalmers, Cuthbertson &

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Synge (1954) showed that heat treatment lessened ammonia production from casein; marked production of ammonia from groundnut meal and moderate production from herring meal were found by Chalmers & Synge (1954*a*) and Annison, Chalmers, Marshall & Synge (1954); the latter authors, and el-Shazly (1952*a*) showed that the ammonia production from several other proteins, including those from hay, concentrates and maize gluten meal, was very much less. The volatile fatty acid production from some of these foodstuffs corresponded roughly to the ammonia production (el-Shazly, 1952*a*; Annison, 1954).

It was the aim of the work described in this paper to obtain more detailed information concerning proteolysis and associated problems in the rumen, using *in vitro* techniques to avoid difficulties due to the continuous influx of soluble nitrogenous compounds in the saliva (McDougall, 1948), and the continuous passage of soluble nitrogenous compounds across the rumen wall (McDonald, 1948) and of soluble and insoluble compounds further down the intestinal tract.

METHODS

Animals. Cheviot ewes or wethers with rumen fistulas were used. Except where otherwise mentioned, the diet was 300 g. hay, 300 g. groundnut meal, 300 g. flaked maize, fed in two portions at 07.30 and 19.30 hr. Samples of rumen liquor were removed, usually at 07.00 hr., and strained through eight thicknesses of surgical gauze.

Materials. Groundnut meal, herring meal, Paisley meal and casein were as described by Annison *et al.* (1954), starch and cellulose as described by Warner (1956). Ardein was a spray-dried groundnut protein (Imperial Chemical Industries Ltd., Nobel House, Stevenston); solutions of it were prepared by shaking with that volume of *c.* 0.2 N-NaOH which, when diluted to the required volume, would be *c.* 0.05 N. Solutions of casein were made in a similar way, but neutralized with $\text{m-H}_3\text{PO}_4$ before making to volume. Groundnut meals I, II, III and IV were four samples that had been subjected to increasing degrees of heat treatment and were made available by the Research Department of J. Bibby and Sons Ltd., Liverpool. Casein hydrolysate was Allen and Hanburys bacteriological product. Phosphate+acetate buffer was prepared by mixing 0.1 N-acetic acid with 0.2 M- Na_2HPO_4 to the desired pH value. Ferrous sulphate solution was freshly prepared 1% (w/v) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ used at a concentration of 1 drop/10 ml. reaction mixture. Cysteine solution was the 10% (w/v) solution of Huhtanen, Rogers & Gall (1952) used at a concentration of 1 drop/10 ml. mixture.

Chemical estimations. Ammonia, volatile fatty acid, and starch were estimated as described by Warner (1956). Total nitrogen was estimated by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943), distilling off the ammonia in the apparatus of Markham (1942) and trapping it in the boric acid indicator reagent of Conway & O'Malley (1942). Non-protein nitrogen was estimated by precipitating proteins with trichloroacetic acid (final concentration of 5%, w/v) filtering and estimating the total nitrogen in the filtrate as above. Nitrogen soluble at pH 5 was estimated by adjusting the pH value,

centrifuging and estimating the total nitrogen in the supernatant. Amino nitrogen was estimated by the method of Van Slyke (1929), using either the HCl filtrate used in the ammonia estimations or the supernatant obtained on high-speed centrifugation. Carboxyl nitrogen was estimated according to Van Slyke, Dillon, MacFadyen & Hamilton (1941), using the citrate buffer pH 2.5 and the apparatus of Synge (1951); samples were prepared as for amino nitrogen.

Apparatus. The artificial rumen apparatus described by Warner (1956) was used, with mineral solution II outside the dialysing sac.

RESULTS

Experiments using washed suspensions of rumen micro-organisms

Preliminary work, in large part repeating and confirming that of Sym (1938), showed that washed suspensions of the total microbial population of the rumen were actively proteolytic; toluene had little if any effect on this activity. Sodium sulphide (0.3 %, w/v; Elsdon & Lewis, 1953) either in the fluid used for washing the cells or in that used to suspend them for testing, had no effect on proteolysis; apparently the proteolytic enzymes were relatively stable to aerobic conditions. However, ferrous ion and cysteine (Weil & Kocholaty, 1937) did stimulate proteolysis. About half the proteolytic activity of the micro-organisms taken from the rumen of a sheep on the high protein diet generally used in these experiments (which supports large numbers of protozoa, 10^6 /ml. rumen liquor or more) was due to the protozoa, and about half due to the bacteria. The optimum pH of the bacterial proteolysis was between 6.5 and 7.0. The most active bacterial preparations were obtained 6–10 hr. after feeding the host animal.

The nitrogen partition in the products of proteolysis by toluene-treated rumen bacteria is shown in Table 1. All the casein and just under half the ardein was digested in 46 hr.; this faster rate of attack on casein as compared with ardein was noted in several experiments with different amounts of substrate and other preparations of groundnut meal protein, and is in contrast to the nearly equal rate of attack in the rumen (Annison *et al.* 1954) or by whole rumen liquor *in vitro* (Warner, 1956) where crude groundnut meal was used; no explanation can be offered for this discrepancy. When digestion neared completion, about half the non-protein nitrogen which appeared was in the form of free amino acids (carboxyl-N). The figures are consistent with proteolysis proceeding in the usual way through peptides of decreasing complexity to amino acids. The amount of ammonia produced, as always in the presence of toluene, formed a small proportion only of the products of digestion, of the same order of magnitude as the amide-nitrogen of the protein; this is to be compared with experiments reported below with whole rumen liquor in the artificial rumen apparatus, where ammonia forms a much larger proportion of the proteolysis products. Ferrous ion and cysteine appear to stimulate all stages of proteolysis down to free amino acids.

The nature of the diet had little influence on the proteolytic activity of the

Table 1. *Nitrogen partition in the products of proteolysis by rumen bacteria under toluene*

Rumen liquor was collected 8 hr. after feeding, strained and centrifuged at 1500 r.p.m. for 10 min., discarding the deposit. The supernatant was centrifuged at 2500 r.p.m. for 60 min., and the deposit washed with water. 50 ml. of this washed suspension of rumen bacteria in phosphate+acetate buffer (pH 6.5) with 20 ml. of water, 5 % ardein solution, 2.5 % casein solution or 2.5 % cysteine solution with added cysteine and ferrous sulphate were incubated under toluene at 39°. All values expressed as mg. N/100 ml.

Period of incubation (hr.)	Substrate	'Substrate'-N*			Non-protein nitrogen			NH ₃ -N			Amino-N			Carboxyl-N		
		Found	Decrease		Found	Increase†		Found	Increase†		Found	Increase†		Found	Increase†	
0	Blank	—	—		10.6	—		4.1	—		4.3	—		1.2	—	
	Ardein	220	—		11.4	—		4.4	—		6.0	—		3.3	—	
	Casein	79	—		13.6	—		4.2	—		3.6	—		0.4	—	
	Casein with cysteine, FeSO ₄	87	—		16.5	—		4.1	—		11.2	—		6.3	—	
7½	Blank	—	—		25.0	—		4.2	—		4.3	—		2.4	—	
	Ardein	162	58		63.1	37.3		10.1	5.6		23.6	17.6		14.0	9.5	
	Casein	40	39		49.1	21.1		5.8	1.5		11.8	8.2		7.6	6.0	
	Casein with cysteine, FeSO ₄	37	50		61.8	30.9		6.5	2.3		31.4	20.2		21.1	13.6	
46	Blank	—	—		25.6	—		5.1	—		4.0	—		3.6	—	
	Ardein	125	95		91.8	64.4		20.6	15.2		60.7	55.0		37.9	32.2	
	Casein	0	79		98.6	70.0		14.1	8.9		63.5	60.2		41.0	38.2	
	Casein with cysteine, FeSO ₄	4	83		112.8	83.3		15.0	9.9		78.5	67.6		43.1	34.4	

* Calculated as the difference between the total N and the N soluble at pH 5, less the blank value (experiment had shown both ardein and casein to be less than 2 % soluble at pH 5).

† Corrected for the blank value.

rumen bacteria. Table 2 shows that the rumen bacteria from a sheep fed 800 g. hay only/day were as active as those from a sheep fed the usual groundnut meal + flaked maize + hay ration; similar results were obtained with the rumen bacteria from sheep fed hay and concentrates supplemented with Paisley meal

Table 2. *Proteolysis by washed suspensions of bacteria from the rumens of sheep on different diets*

18 ml. of washed suspensions of bacteria in phosphate + acetate buffer (pH 6.5) prepared as before, were incubated for 42 hr. at 39° with 3 ml. of water, 2.5 % casein or 5 % ardein under toluene with added cysteine and FeSO₄. Results are expressed in mg. N/100 ml.

Diet of sheep	Substrate	'Substrate'-N*			Amino-N			Ammonia-N		
		Initial	Final	Decrease	Initial	Final	Increase†	Initial	Final	Increase†
Hay	Blank	—	—	—	3.2	10.0	—	3.0	3.9	—
	Casein	37.1	4.9	32.2	2.9	31.5	21.8	3.0	8.5	4.6
	Ardein	102.4	72.0	30.4	3.8	30.7	20.1	3.3	10.6	6.4
Groundnut meal	Blank	—	—	—	7.8	16.7	—	8.5	10.6	—
	Casein	39.6	8.6	31.0	7.8	43.4	26.7	8.8	16.2	5.3
	Ardein	96.5	60.5	36.0	8.9	41.6	23.8	8.8	19.2	8.3

* Calculated as before (footnote to Table 1).

† Corrected for blank values.

Table 3. *Effect of heat treatment on groundnut meals*

The four groundnut meals, subjected to increasing degrees of heat treatment, were extracted with 0.05 N-NaOH and the solutions adjusted to contain 54 mg. N/10 ml. 20 ml. of a washed suspension of bacteria from the rumen of a groundnut meal-fed sheep in phosphate + acetate buffer (pH 6.5) were added and the whole incubated with cysteine and ferrous ions under toluene at 39° for 42 hr.; the results are given in columns 5-7. 1 g. of each of the finely powdered meals were suspended in 5 ml. phosphate + acetate buffer (pH 6.5) and mixed with 15 ml. of rumen liquor that had been centrifuged at 1500 r.p.m. for 5 min. to remove protozoa. The mixtures were incubated under toluene for 42 hr. at 39° and the results given in columns 8-10. All results are expressed in mg. N/100 ml.

Ground-nut meal	Total N (%)*	Salt-peptizable N (%)*	Alkali-soluble N† (% of total N)	'Substrate' N decrease‡	Soluble fraction		Whole groundnut meal		
					Amino-N increase	Ammonia-N increase	'Substrate' N decrease‡	Amino-N increase	Ammonia-N increase
I	8.80	74	98	53.8	39.6	8.7	40.0	30.3	6.8
II	8.65	56	89	46.6	38.4	9.0	35.9	25.6	4.4
III	8.56	37	88	52.1	45.3	10.4	25.5	19.2	4.0
IV	8.43	29	64	45.0	37.1	9.2	17.5	13.3	4.4

* Figures supplied by the Research Department of J. Bibby and Sons, Ltd., Liverpool; 'salt-peptizable nitrogen' is the percentage of the total N soluble in 5 % NaCl.

† 2.0 g. of each sample suspended in 0.05 N-NaOH, allowed to stand overnight and centrifuged; the N content in the supernatant solution was then determined.

‡ Calculated as before (footnote to Table 1).

or herring meal. This independence of the proteolytic activity of the bacteria on the presence of readily attacked protein in the diet is in contrast to the dependence of deaminase activity as found by el-Shazly (1952*b*).

The four groundnut meals (subjected to increasing degrees of heat treatment; supplied by J. Bibby and Sons Ltd.) were tested for their *in vitro* digestibility, with results as shown in Table 3. That fraction of the nitrogen of these

groundnut meals which was extractable with 0.05 N-NaOH was attacked by rumen bacteria to very much the same extent; ardein was however attacked more readily than these extracts. The digestibility of the powdered meals suspended in rumen liquor freed from protozoa, in the presence of toluene, was quite small, only about 10 % of the initial 'substrate'-nitrogen disappearing in the most readily attacked specimen. Thus, increasing degrees of heat treatment decreased the solubility in both dilute alkali and in salt solutions, and also decreased the susceptibility to proteolysis, in much the same way as heat treatment decreased the ammonia production from casein in the rumen *in vivo* in the experiments of Chalmers *et al.* (1954).

Experiments with the artificial rumen apparatus

Warner (1956) showed that casein was digested in an artificial rumen apparatus at a rate of about 0.3 g. N/l. rumen liquor/hr. as measured by the time at which the excess ammonia production due to casein ceased. At the completion of digestion, 12.5 m-equiv. N/100 ml. of casein produced, in eleven experiments, 6.5 ± 0.9 m-equiv. ammonia/100 ml. (range 5.2–8.0) and 6.5 ± 1.1 m-equiv. volatile fatty acid/100 ml. (range 5.0–8.1), allowing for blanks in each case. That is, about half the added casein-N was recovered as ammonia-N; since the excess of non-protein nitrogen over ammonia-N was little greater at the end of the experimental period than at the beginning, it is presumed that much of the remaining half of the casein-N was utilized for microbial growth. The carbon chain of the amino acids was apparently also utilized for growth, otherwise there would have been a marked excess of volatile fatty acid over ammonia, but the figures do not distinguish between use as energy source with production of CO₂, and use as cell material.

Starch has been shown to lower ammonia production by rumen micro-organisms *in vitro* as *in vivo* (Warner, 1956). This effect, as Table 4 shows, is shared by other polysaccharides and polysaccharide-rich foodstuffs. It does not appear to be due to any inhibition of proteolysis, as in these experiments rapid ammonia production from casein ceased at approximately the same time in the presence and in the absence of polysaccharide, nor does it appear to be due to any effect on deamination, since Table 5 shows that amino acids placed in the artificial rumen disappeared, if anything, more rapidly in the presence than in the absence of starch, with a lower production of ammonia. The remaining possibility, that the effect is due to increased utilization by the rumen micro-organisms of some breakdown product of the protein, has been commonly accepted to explain the analogous *in vivo* findings, but direct evidence has been difficult to obtain. No significant increase in numbers of either protozoa, selenomonads or bacteria (counted by methods of Warner, 1956), was found over a period of 7 hr. in an artificial rumen containing starch and casein as compared with one containing casein alone.

When using in the artificial rumen an inoculum of rumen liquor taken from a sheep fed hay alone, instead of from one fed the standard groundnut meal diet, several effects were noted (Table 6). The continuing production of ammonia in the absence of added substrate was lower, that of volatile fatty acid very

Table 4. *Volatile fatty acid production from polysaccharides and polysaccharide-rich foodstuffs, and their effect on ammonia production by rumen micro-organisms in the artificial rumen*

50 ml. rumen liquor were placed in the artificial rumen apparatus, with or without 1.0–1.4 % casein, and incubated for 8 hr. at 39°, and the increase in volatile fatty acid production and the decrease in ammonia production due to supplements as indicated, as compared with controls, was measured.

Supplement (%, w/v)	Increase in volatile fatty acid (m-equiv./100 ml.)		Decrease in ammonia (m-equiv./100 ml.)	
	Alone	With casein	Alone	With casein
2.4 starch*	9.5	12.2	4.3	3.4
1.8 starch*	8.4	8.0	1.5	1.7
4 cellulose*	2.2	2.8	0.7	0.8
2 cellulose*	1.2	0.6	0.2	0.5
4 hay	5.1	5.0	1.3	2.3
3 concentrates†	7.2	8.1	0.0	1.3

* Tests done at widely differing times so that between one experiment and the next changes in microbial activity may well have taken place.

† The Mixed Meals I of Chalmers, Cuthbertson & Synge (1954).

Table 5. *Deamination of amino acids and the effect of starch*

Artificial rumens containing 15 ml. mineral solution, 40 ml. rumen liquor and either 0.4 g. casein hydrolysate, 1.5 g. starch, both or neither were set up. The two experiments, with and without starch, were done on different days and are not strictly comparable, as there may have been some small variation in microbial activity. At the end of 4 hr. the following changes were noted in the artificial rumens containing casein hydrolysate, correcting for blank values:

	Decrease in carboxyl-N (mg./100 ml.)	Increase in ammonia-N (mg./100 ml.)
Without starch	23.0	27.2
With starch	31.1	23.2

Table 6. *The influence of the diet on starch and casein digestion in the artificial rumen*

The experiment using rumen liquor from a sheep fed the standard groundnut meal diet is the same as that reported in Table 4 of Warner (1956), where 1.8 % starch and 1.0 % casein were added and incubation continued for 9 hr. In the experiment using rumen liquor from a sheep fed 900 g. hay/day, 2.2 % starch and 1.2 % casein were added and incubated for 8 hr.

		Substrate in artificial rumen			
		None	Starch	Casein	Starch and casein
Diet of sheep	Groundnut meal	Decrease in starch concn. (g./100 ml.)			
	Hay				
Groundnut meal	Decrease in ammonia concn. (m-equiv./100 ml.)	0.2	1.1	0.2	1.4
	Hay	0.0	0.4	0.0	0.3
Groundnut meal	Increase in non-protein nitrogen concn. (m-equiv./100 ml.)	2.5	1.1	8.4	6.7
	Hay	0.8	0.1	2.5	2.3
Groundnut meal	Increase in volatile fatty acid concn. (m-equiv./100 ml.)	3.4	2.6	8.7	7.7
	Hay	0.9	0.3	8.1	6.8
Groundnut meal	Increase in volatile fatty acid concn. (m-equiv./100 ml.)	10.6	19.0	18.7	26.7
	Hay	0.3	1.8	2.0	4.1

much lower, with the rumen liquor from the hay-fed sheep. Ammonia formed a much smaller proportion of the non-protein nitrogen produced from casein, though this latter figure was only slightly altered. This is consistent with the high proteolytic activity of the bacteria from the rumen of a hay-fed sheep reported above and el-Shazly's (1952*b*) finding that their deaminating power was markedly decreased. Further examination of rumen liquor from a hay-fed sheep digesting casein in the artificial rumen apparatus showed that the concentration of free amino acids (carboxyl nitrogen) was little higher than was found when using rumen liquor from sheep fed the standard groundnut meal diet, i.e. up to about 10 mg. N/100 ml.; but amino nitrogen was found at

Table 7. *Effect on proteolysis of removal of protozoa*

Rumen liquor from sheep fed the standard groundnut meal diet or 900 g. hay/day was centrifuged at a speed just sufficient to remove all the protozoa from the preparation. This supernatant and the original rumen liquor were then incubated for 9 hr. in the artificial rumen with or without 1.4% added casein (12.5 m-equiv. N).

Diet of sheep	Preparation	Increase in ammonia concn. (m-equiv./100 ml.)		Increase in non-protein nitrogen concn. (m-equiv./100 ml.)		Increase in volatile fatty acid concn. (m-equiv./100 ml.)	
		Blank	Casein	Blank	Casein	Blank	Casein
Groundnut meal	Whole	2.66	8.58*	2.15	10.92	3.4	8.4
	Centrifuged	0.80	3.26	1.42	6.95	0.6	2.7
Hay	Whole	0.63	3.49	2.08	8.58	2.1	3.3
	Centrifuged	0.18	1.90	0.48	7.60	1.1	1.5

* This is the only case where the curve for ammonia production flattened out, suggesting completion of digestion within 9 hr.

a concentration roughly equal to the ammonia nitrogen, suggesting that perhaps peptidase activity was also somewhat diminished. It is of interest that Sym (1938), using rumen liquor obtained from the slaughter-house from animals of unknown dietary history, found peptidase activity to be weak. Warner (1956) noted the dependence of the rate of starch digestion on the diet; it should also be noted that the ratio starch digested: volatile fatty acid produced (see Table 6) was higher with rumen liquor from the hay-fed sheep, though it is uncertain whether this was due to a lowered overall rate of digestion with a higher proportion of intermediate products (e.g. dextrins, etc.) or to the existence of alternative routes of metabolism, such as to lactic acid.

When rumen liquor was centrifuged to remove protozoa, the rate of proteolysis and the rates of production of ammonia and volatile fatty acid in the absence of added substrate were all decreased, as shown in Table 7. However, the most marked decrease, whether the sheep supplying the rumen liquor inoculum was fed hay only or the standard groundnut meal diet, was in the rate of production of ammonia in the absence of added substrate. Since the rumen liquor inocula were removed from the animals 12 hr. after feeding, by which time one would expect ammonia production from the ration to have ceased, and since in any case ammonia production from hay alone was negligible

in vitro, it is thought that this ammonia production in the absence of added substrate was not due to residual food particles but to the activity of the protozoa, presumably as the end product of their endogenous nitrogen metabolism. The lower rate of production of ammonia in the absence of added substrate in the rumen liquor from the hay-fed sheep is consistent with the much smaller number of protozoa present (though the numbers of holotrich protozoa were similar in the hay-fed and the groundnut meal-fed sheep, the oligotrich protozoa, mostly *Entodinia* spp., were about one-tenth as numerous in the hay-fed sheep). Similar conclusions in the case of the volatile fatty acid cannot so safely be drawn, as there would still be much undigested polysaccharide material in the rumen 12 hr. after feeding that would be capable of producing volatile fatty acid and would be at least partially removed by centrifugation.

Experiments with cell-free enzyme preparations

Schlottke (1936) prepared from rumen micro-organisms cell-free glycerol extracts which showed considerable proteolytic activity; he did not, however, give details of technique for preparing the extracts; attempts to repeat his work were unsuccessful. However, the technique given by Sym (1938) for preparing cell-free extracts of acetone powders of rumen micro-organisms proved readily repeatable. Active proteolysis was obtained with simple acetone-dried powders of holotrich protozoa, oligotrich protozoa, selenomonads and bacteria from hay-fed sheep and from groundnut meal-fed sheep, by extracting with water or dilute slightly alkaline buffer. (The separation of the various fractions of rumen micro-organism, done by differential centrifugation, was not complete so that unequivocal proof of proteolytic activity by the larger micro-organisms is lacking.) When these extracts were dialysed first against tap water, then against distilled water, freeze-dried and, as needed, re-dissolved, little activity was lost. No stimulation by ferrous ion or cysteine or a mixture of both was noted, in contrast with the washed suspensions as reported above. Similarly, there was no stimulation by calcium ion or inhibition by citrate as found with extracts of other proteolytic micro-organisms by Gorini (1950).

Comparison of preparations of rumen micro-organisms

The various preparations described above, and some others, were compared using as source of organisms one sample of rumen liquor obtained from a sheep fed the standard groundnut meal diet. Preparations were made as follows:

- (1) Whole rumen liquor in the artificial rumen apparatus of Warner (1956).
- (2) Whole rumen liquor incubated under a 1 in. layer of liquid paraffin to secure anaerobiosis, following el-Shazly (1952*b*).
- (3) The clear supernatant obtained by centrifuging the rumen liquor at 4900 r.p.m. at 2° for 90 min., incubated under toluene.
- (4) The deposit obtained from the above centrifugation was re-suspended in the acetate buffer of Heald & Oxford (1953), centrifuged at 700 r.p.m. at 2° for 10 min. and this process repeated three times. Then a suspension was made in a volume of buffer approximately equal to the volume of the original sample of

rumen liquor. Microscopical examination showed mainly protozoa with only a small number of selenomonads and some bacteria. This suspension was tested under a layer of toluene and also in a closed vessel through which a slow current of nitrogen containing 5 % carbon dioxide (British Oxygen Co. Ltd.) was passing.

(5) The supernatant fluids obtained in the centrifugations of section 4 were centrifuged at 4500 r.p.m. at 2° for 90 min. the deposit washed twice and suspended in acetate buffer. Microscopical examination showed bacteria and selenomonads with hardly any protozoa. This suspension was also incubated under toluene and in an atmosphere of nitrogen + carbon dioxide.

(6) To samples of the protozoa-rich fraction and of the bacteria-rich fraction obtained above, about 4 vol. of acetone at -20° were added; after standing a few minutes the suspensions were filtered and the deposits washed with acetone, acetone + ether and ether, all at about -10°, and allowed to dry. These acetone powders were then extracted twice with *c.* 0.001 M-Na₂HPO₄, the centrifuged extracts dialysed against running tap water overnight and against three changes of distilled water for 2 hr. each, and then freeze-dried. The resulting powder was suspended in a volume of phosphate + acetate buffer pH 6.5, corresponding to about half the volume of the original washed suspension or the original rumen liquor, and centrifuged, the clear supernatant fluid being tested under a layer of toluene.

(7) Further samples of both microbial fractions were frozen at -20° overnight, allowed to thaw to room temperature, frozen again for a few hours and thawed again. The material was then centrifuged and the supernatant fluid dialysed, freeze-dried and taken up in buffer as above, testing for activity under toluene.

Table 8. *Comparison of preparations of rumen micro-organisms*

Each preparation was incubated for 5 hr. at 39° with a solution of casein at a final concentration of 0.8 % (110 mg. N/100 ml.). For methods of making preparations see text. Values are corrected for volume changes during preparation, and blanks have been subtracted.

Preparation	Total N content (mg./100 ml.)	Increase in ammonia concn. (mg. N/100 ml.)	Increase in non-protein nitrogen (mg. N/100 ml.)
Rumen liquor in artificial rumen	265	45.6	81
Rumen liquor under paraffin	265	21.7	96
Supernatant under toluene	60	0.6	12
Bacterial fraction, under N ₂ + CO ₂	44	9.5	46
Protozoal fraction, under N ₂ + CO ₂	165	6.2	39
Bacterial fraction under toluene	44	0.5	27
Protozoal fraction under toluene	165	5.2	39

These suspensions and extracts were then tested for proteolytic activity against casein, with results as shown in Tables 8 and 9. From examination of the non-protein nitrogen figures in Table 8, it seems probable that the higher value for the rumen liquor under paraffin as compared with the artificial rumen was due to better utilization of the products of proteolysis by the micro-organisms under the more physiological conditions of the artificial rumen; it

should be noted that the excess of non-protein nitrogen over ammonia-N largely disappeared on continuing incubation until digestion was complete. The sum of the activities of the bacterial fraction and the protozoal fraction (incubated under nitrogen) and of the supernatant fluid was approximately equal to the activity of the whole rumen liquor incubated under paraffin. Toluene caused some inhibition of the bacterial fraction, though not of the protozoal fraction, as compared with the activity under nitrogen. This inhibition was rather more than had been found in previous experiments, but it largely disappeared when

Table 9. *Comparison of cell-free extracts*

Extracts were prepared as described in the text from the bacterial and protozoal fractions used in Table 8. They were incubated under toluene at 39° with casein at a final concentration of 0.8 % (110 mg. N/100 ml.) for the times indicated.

Preparation	Total N content (mg. N/100 ml.)	Period of incubation (hr.)		
		5	24	72
		Increase* in non-protein nitrogen concn. (mg. N/100 ml.)		
Bacteria; acetone powder	4.2	3.3	17.5	41.6
Bacteria; frozen and thawed	2.5	1.7	4.2	7.5
Protozoa; acetone powder	39.7	20.0	60.0	73.1
Protozoa; frozen and thawed	50.0	11.8	41.6	60.0

* Blank values remained substantially constant at a very low figure throughout.

incubation was continued for 24 hr. Ammonia production appeared to be more sensitive to unphysiological conditions than was the production of non-protein nitrogen, and toluene abolished almost completely the ammonia production due to bacteria, though it had a much smaller effect on that due to protozoa. In the preparation of the cell-free extracts, activity was considerably decreased, particularly with the bacterial fraction. The frozen and thawed preparation would seem to be one where much active material could be extracted from the protozoa, and only a very little from the bacteria; this is consistent with the known susceptibility of these types of micro-organism to lysis by this method.

DISCUSSION

There is good reason to believe that proteolysis in the rumen takes place in the usual way, through peptides of decreasing chain length to free amino acids which are then deaminated to ammonia. Toluene-treated washed suspensions of rumen bacteria, by permitting free amino acids to accumulate in considerable quantity, showed this sequence particularly well. In the artificial rumen, and hence, since Warner (1956) showed that this artificial rumen allowed many true rumen activities to occur normally, probably *in vivo* also, about half the nitrogen of added casein was recoverable as ammonia, and it is presumed that the remaining half was utilized for microbial growth, though whether or to what extent peptides, free amino acids or ammonia were so utilized is unknown. Direct counts of several groups of micro-organisms showed that microbial growth occurred in the artificial rumen (Warner, 1956).

At least half the proteolytic activity appears to be due to the bacteria of the rumen. The activity of washed suspensions of rumen bacteria was stimulated by ferrous ion and cysteine, resembling in this respect the anaerobic clostridia (Weil, Kocholaty & Smith, 1939), though this stimulation did not seem to occur with cell-free extracts of the bacteria. Identification of proteolytic bacteria from the rumen is incomplete. Van der Wath (1948) inserted casein into the rumen enclosed in a silk bag, and after incubation noted increased concentrations of Gram-positive cocco-bacilli, bacilli and large cocci in association with the protein; the present writer has confirmed this observation. Gutierrez (1953) isolated in pure culture proteolytic Gram-positive bacteria, and Bryant & Burkey (1953*a, b*) isolated proteolytic Gram-positive and weakly proteolytic Gram-negative bacteria from the rumen. Appleby (1955) isolated several proteolytic *Bacillus* spp., and, in lesser numbers, other proteolytic bacteria from the rumen contents of the sheep (fed the standard groundnut meal diet), which was used by the present author.

Demonstration of proteolytic activity by the rumen protozoa is more difficult. It is impossible to get a preparation of protozoa completely free from external bacteria, particularly when using the viscous rumen liquor associated with a high protein diet. In addition, many protozoa have bacteria within them, fixed to plant particles or free. However, provided the bacteria present in the preparations used to make both washed suspensions and cell-free extracts of rumen protozoa were not very much more active in proteolysis than the generality of rumen bacteria, the protozoa must have been responsible for part of the activity. This is probably best indicated by the experiment where simple freezing and thawing released a considerable amount of active protease from a suspension of rumen protozoa contaminated with a few bacteria, but very little from a similar suspension rich in rumen bacteria only.

Proteolysis and ammonia production by the protozoa were less sensitive to inhibition by toluene than the same reactions by the bacteria. Ammonia production in particular appeared to occur by different mechanisms in the bacteria and the protozoa, and it is suggested that much of the continuing ammonia production in the rumen in the absence of readily attacked protein might be due to the endogenous metabolism of rumen protozoa; ammonia is known to be the major end product of nitrogen metabolism in some free-living ciliate protozoa (Kidder & Dewey, 1951).

El-Shazly (1952*b*) found that the capacity of rumen bacteria to deaminate amino acids depended on the presence in the diet of the host animal of a readily attacked protein. This does not appear to be the case for the power to cause proteolysis, except perhaps in so far as a high protein diet will usually support a more numerous microbial population. Washed suspensions of rumen bacteria, when used at similar concentrations, caused proteolysis at similar rates whether the diet of the animal from which they came contained hay alone or ample amounts of either readily soluble or insoluble protein. In the artificial rumen with whole rumen liquor somewhat more difference was noted, but the proteolytic activity of rumen liquor from a hay-fed sheep was much more like that from a groundnut meal-fed sheep than was the case of deaminative

activity as found by el-Shazly (1952*b*) and shown in the present experiments by the much lower proportion of ammonia in the products of digestion of casein by the rumen micro-organisms of the hay-fed sheep.

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Reversion of a Pleuropneumonia-like Organism to a *Corynebacterium* during Tissue Culture Passage

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SUMMARY: A strain of a pleuropneumonia-like organism (PPLO) isolated from urethral exudate from a case of non-specific urethritis was studied in HeLa cell tissue cultures. Although the organisms entered the cell cytoplasm, they did not produce marked damage or proliferate luxuriantly until filtrate from a broth culture of *Staphylococcus pyogenes* or yeast extract was added to the infected tissue cultures. The organisms subsequently isolated from tissue cultures initially inoculated with PPLO and yeast extract showed conversion from PPLO form to L form of growth. Further culture of the L form, especially with the aid of mucin, resulted in conversion of the L form to a corynebacterium. This corynebacterium was indistinguishable culturally, biochemically and serologically from a corynebacterium isolated on rabbit blood agar plates which had been inoculated with a portion of the original urethral exudate from the same case of non-specific urethritis. The view is expressed that other human genital strains regarded at present as PPLO may be found to be L forms of *Corynebacterium* spp. The criteria for identification of PPLO and L forms are discussed.

During recent years pleuropneumonia-like organisms (PPLO) have been isolated with increasing frequency from the human urethra in the presence and in the absence of various inflammatory processes (Harkness, 1950; Melén & Linnros, 1952; Nicol & Edward, 1953). The pathogenic significance of the PPLO in genital infections, however, is controversial, and most investigators emphasize the need for more information about the organisms themselves before an aetiological role in genital diseases can be attributed to them. Keller & Morton (1954) reported that several human genital strains of PPLO produced no pathological manifestations in the developing chick embryo. No reports have come to our attention on the action of human PPLO on human cells in tissue culture, although Edward (1952) mentioned that tissue cultures might be useful tools for investigating the pathogenicity of PPLO. This investigation was undertaken, therefore, to obtain basic morphological data on the behaviour of a human urethral strain of PPLO in cultures of human cells. The manifestation of any pathogenic effects by the organisms upon the cells was sought. For comparison, the behaviour in tissue culture of a presumably non-pathogenic corynebacterium from the same source as the PPLO was observed. The role of secondary factors which might possibly alter the behaviour of the PPLO in the tissue cell environment was also investigated. The secondary factors chosen for

the study were (a) the filtrate from cultures of *Staphylococcus pyogenes*, another presumably non-pathogenic organism isolated from the same source as the PPLO, and (b) yeast extract as a substitute for *S. pyogenes* filtrate. The results of these studies brought to light an unsuspected relationship between the PPLO and the corynebacterium.

METHODS

Source and collection of organisms. The organisms used in this investigation were freshly isolated from urethral exudate from a patient with non-specific urethritis. Since this disease itself remains ill-defined, choice of the case was based on the criteria for differential diagnosis of non-specific urethritis outlined by Graham (1954). Although the patient had a past history of gonorrhoea, he had been successfully treated with penicillin. Cultures and smears of the exudate were negative for gonococci at the time of this study. Hanging-drop examination of the urethral specimen excluded *Trichomonas* infection, and serological examination of the patient's blood gave negative results in tests for syphilis.

The urethral exudate was collected on two cotton swabs. One swab was placed in a small amount of ascitic fluid + veal infusion broth containing 150 units penicillin/ml. and was cultured for PPLO and for L forms, i.e., the pleuropneumonia-like growth phase of ordinary bacterial genera. The other swab was kept moist with physiological saline solution and was cultured for bacterial species other than PPLO and L forms. The exudate was cultured within 3 hr. of its collection.

Species of organisms used. The three organisms recovered from the urethral exudate and used in this study were: PPLO, a *Corynebacterium* sp., and *Staphylococcus pyogenes* var. *albus*. The PPLO was a strain which did not ferment glucose and grew poorly if at all on media enriched with horse serum. It was identified as a PPLO rather than an L form on the basis of the cultural and morphological characteristics listed in Table 1. The *Corynebacterium* sp. was a strain non-toxigenic for guinea-pigs and rabbits; it fermented glucose but not maltose or sucrose, was catalase-positive, reduced nitrate to nitrite, and did not hydrolyse urea or produce change in litmus milk. On tellurite agar it formed small, black, shiny, raised, smooth colonies.

Culture media. The medium used for isolation and maintenance of the corynebacterium and *Staphylococcus pyogenes* strains was trypticase soy agar containing 5 % (v/v) rabbit blood. Incubation of the *Corynebacterium* sp. was carried out in a candle jar.

The medium used for the PPLO was veal infusion agar (pH 7.6-7.7) containing 20 % (v/v) human ascitic fluid. For primary isolation (but not thereafter) 1 drop of a penicillin solution (10,000 units/ml.) was placed in a cup on the agar plate after inoculation of the specimen to prevent overgrowth by bacterial colonies. Veal infusion broth containing 20 % (v/v) human ascitic fluid was used whenever fluid cultures were desired.

Tissue cultures. The human epidermoid carcinoma cells (strain HeLa, from

Microbiological Associates, Bethesda, Maryland, U.S.A.; Scherer, Syverton & Gey, 1953) were cultured and maintained as described by Warren, Wittler & Vincent (1955). Before inoculation with any of the strains of organisms, the tissues were washed repeatedly with maintenance fluid to remove antibiotics originally incorporated in the growth fluids. Antibiotics were never added thereafter to the tissue cultures.

Staining methods. Conventional bacterial forms were stained with methylene blue and by the Gram method.

PPLO and L forms were stained by the method of Klieneberger-Nobel (1950) using impression preparations and Giemsa stain. The method of Dienes (1939, 1942), using direct staining of colonies on agar with methylene blue and azure stain previously dried on a coverslip, was combined with examination by phase contrast using a microscope with a long working distance condenser and an oil-immersion objective.

Tissue cells were stained *in situ* after removal of the maintenance fluids. The cells were rinsed rapidly with cold M/150-phosphate buffer (pH 7.2), fixed in cold methanol for 4 min., then rinsed again with buffer. When dry, the cells were stained in a 2% (v/v) solution of Giemsa for 30 min., rinsed once with buffer, and allowed to dry.

Serological methods. The *corynebacterium* strain used for rabbit immunization was grown in veal infusion broth containing 5% (v/v) rabbit serum. Growth was sedimented by centrifugation, resuspended in 2% (v/v) formolized saline, and allowed to stand at 5° for 24 hr. The organisms were then washed three times in saline, resuspended in 0.2% (v/v) formolized saline, and adjusted to match a no. 5 McFarland density standard. Rabbits were injected with increasing doses of the antigens on 4 successive days each week for 3 weeks. Agglutination tests were incubated at 52° for 18 hr. and then read.

The PPLO used for rabbit immunization was grown in ascitic fluid + infusion broth. The growth was sedimented by centrifugation, washed and resuspended in saline, and adjusted to match a no. 3 McFarland density standard. Rabbits were injected with 2 ml. of freshly prepared living antigen on 3 successive days each week for 3 weeks. Agglutination tests were carried out by the method of Edward (1950).

RESULTS

Behaviour of PPLO in HeLa cultures

A detailed study of the behaviour and appearance of the PPLO strain in HeLa cells was made, and evidence for all pathological changes in the infected tissue cells was noted. Multiplication of the PPLO was determined by subculture to agar plates. The appearance in tissue culture of the PPLO and of the HeLa cells was examined microscopically after staining with Giemsa solution.

An ascitic fluid + infusion broth culture of the PPLO was inoculated and passed serially every 6 days in HeLa cell cultures. Ordinarily 0.2 ml. of undiluted inoculum was used for each tissue culture plate during these and all following experiments. The PPLO were found in the cytoplasm but appeared

to cause little damage to the HeLa cells. By the second or third serial passage, the PPLO could no longer be recovered from the tissue cultures by subculture to agar. Small red granules and bodies similar to those described by Harkness (1950) were found in the Giemsa-stained HeLa cells infected with PPLO. These inclusions persisted during several further serial passages, but then were no longer discernible. Under these experimental conditions the PPLO itself did not induce gross pathological changes in the tissue cells, but seemed instead to have been destroyed or possibly lost during continued passage in tissue cultures.

Behaviour of Corynebacterium sp. in HeLa cultures

The ability of the corynebacterium to produce gross damage in the tissue cells was then studied for comparison with the PPLO. The procedures followed were similar to those previously used for observation of the PPLO strain.

The corynebacterium also appeared in the cytoplasm of the HeLa cells without producing marked damage. Agar subculture from the second or third tissue culture passage yielded no growth of the corynebacterium. By the fourth or fifth tissue passage, the infected Giemsa-stained HeLa cells showed only very few highly pleomorphic rods. It was, however, noted that inclusions resembling L forms of bacteria (Harkness, 1950; Wittler, 1952) were present in these tissue cells. These experiments indicated that the corynebacterium itself did not induce gross pathological changes in the tissue cells. There was, however, definite evidence that the bacterium was not at once destroyed or lost, but that it underwent a morphological change while in the tissue cultures. This type of change in the morphology of an organism during its sojourn in living cells had previously been described for *Haemophilus pertussis* in the mouse lung (Wittler, 1952).

Effect of the addition of Staphylococcus pyogenes filtrate to PPLO-infected HeLa cells

Klieneberger-Noble (1954) pointed out that it might '...be profitable to search for a second factor or second infective agent in natural diseases associated with PPLO, when a causal relationship between organisms and the disease has not been established.' In view of the lack of evidence in previous experiments for direct destructive action by the PPLO on the HeLa cells, the possibility of enhancing the pathogenicity or altering the behaviour of the PPLO in the cells in the presence of a secondary factor was investigated.

A bacteria-free infusion broth filtrate, made from 24 to 48 hr. cultures of the *Staphylococcus pyogenes* strain, was tested for its ability to supply a possible secondary factor. *S. pyogenes* was chosen since it was present together with the PPLO in the urethral exudate, and since members of this genus are quite commonly found in such exudates. The filtrate was added in 0.2 ml. amounts to HeLa cultures infected with second or third serial tissue culture passage PPLO. Within 48 hr. after addition of the *S. pyogenes* filtrate, the HeLa cells were laden with structures resembling PPLO, and PPLO grew luxuriantly

when such cells were subcultured on agar. Such HeLa cells, inoculated with PPLO together with *S. pyogenes* filtrate, showed very extensive damage. Relatively moderate damage was produced in control cells treated with the filtrate alone. Veal infusion broth alone, tested in other sets of control cells, did not produce cell destruction. Infusion broth added to PPLO-infected cells did, however, enhance the growth of the PPLO sufficiently to permit recovery of at least a few organisms on the agar subcultures. These results indicated that factors in *S. pyogenes* broth filtrate were capable of altering the behaviour of PPLO in HeLa cultures, so that luxuriant growth of the PPLO occurred and concomitantly the tissue cells were destroyed.

Effect of the addition of yeast extract to PPLO-infected HeLa cells

Edward (1947) reported that yeast extract, as well as staphylococcal culture filtrate, added to artificial culture media enhanced the growth of certain strains of PPLO. The ability of yeast extract to replace *Staphylococcus pyogenes* filtrate in PPLO-infected tissue cultures was, therefore, tested. It was found that the addition of 0.5 % (v/v, expressed as final concentration throughout this report) yeast extract caused an increase in growth of the PPLO comparable to that which occurred when *S. pyogenes* filtrate was used. Furthermore, partial destruction of the HeLa cells also occurred under these conditions, although this amount of yeast extract caused only slight damage to uninfected HeLa cells. Increasing the yeast extract to 4 % resulted in total destruction of the HeLa cells but still allowed a great increase in growth of the PPLO to take place.

The PPLO which were subcultured to agar from the yeast extract-treated tissue cells showed a striking change in colonial form. Originally the PPLO colonies were small and discreet, grew deeply into the agar, showed a typical dense centre with lighter periphery, and were composed mainly of tiny granule-like particles and small vacuoles. Pl. 1, fig. 1, illustrates the typical morphological features of a stock transfer of the PPLO strain on agar before passage in tissue culture. The altered PPLO colonies which developed after tissue culture passage in the presence of yeast extract were quite large and spreading. They did not grow as deeply into the agar and often did not show a well-defined centre. They were composed of large globules and vacuoles, large and small bodies, and masses of amorphous material in addition to the granule-like particles. These colonies resembled L colonies (similar to large L colony in Pl. 1, fig. 2) rather than classical PPLO colonies (Table 1). Furthermore, the infected Giemsa-stained HeLa cells treated with yeast extract showed, in addition to the small red granules and bodies, various inclusions resembling L forms and occasionally small rods morphologically similar to the corynebacterium. Pl. 1, figs. 3 and 4, illustrate infected Giemsa-stained HeLa cells treated with yeast extract. The cells were photographed before maximum cytoplasmic destruction had occurred. At this stage the PPLO had grown luxuriantly, and large numbers of the organisms were packed in the cell cytoplasm. The small individual granules and the pleomorphic elements resembling L forms and rod forms all of which composed the clusters may be best

distinguished lying free of the cytoplasm or near the edges of the cells. Yeast extract and *Staphylococcus pyogenes* filtrate appeared to be equally capable of enhancing the growth of PPLO in tissue culture and of causing tissue cell damage. The yeast extract, however, brought about a striking morphological change in the PPLO. Whether the factor responsible for this change in the PPLO is present also in *S. pyogenes* filtrate remains to be investigated.

Table 1. *Cultural and morphological characters used for distinguishing between PPLO and L forms of bacteria*

The distinctions tabulated here are based on published data from Klieneberger-Nobel (1954), Edward (1954), and on personal observations.

Criteria	PPLO	L forms
Growth in broth	Very faint turbidity; very fine colony clumps visible only with magnification	Light turbidity; larger colony clumps, 0.5–1.0 mm., easily visible
Growth on agar	Colonies small (approximately 0.1–0.3 mm.), round, circumscribed, transparent, with fine surface markings Distinct central 'button' Grow deeply into the agar	Colonies larger (approximately 0.5–1.0 mm.), frequent irregularity in shape, more opaque, with coarser surface markings Central 'button' not always well defined May not grow as deeply into the agar
Relative stability upon initial isolation	Cultures established easily on agar and retain typical colonial form after subculture	Cultures established with difficulty on agar and especially in broth because of frequent instability during early passages; may die off or revert to bacterial form
Microscopic appearance of elements composing colonies	Minimal reproductive units regularly arranged and lying more or less in one plane Bodies frequently fairly homogeneous in shape and size Relatively little amorphous material present 'Myelin' structures not abundant or conspicuous by darkground microscopy	Minimal reproductive units irregularly arranged, often in clumps, and lying in various planes Bodies frequently highly pleomorphic in shape with large variation in size Much amorphous material frequently present 'Myelin' structures frequently abundant and conspicuous by darkground microscopy

Reversion of PPLO to a corynebacterium

The foregoing experiments showed that under certain conditions in tissue culture both the corynebacterium and the PPLO could assume the L form, and that the PPLO could possibly appear in a bacillary form. It was, therefore, necessary to ascertain whether the PPLO was, in fact, related to, or perhaps was the L form of the *Corynebacterium* sp.

The PPLO subculture used in this particular test was one which had never been exposed to a tissue culture environment but had been transferred regularly on agar for many months. A broth subculture of the PPLO was inoculated

into HeLa cultures, and the fluids were harvested from the tissue cultures after 6 days. A second serial passage was performed and again harvested on the sixth day. Agar subcultures at this point yielded a light growth of typical PPLO colonies. The harvest material was passed a third time in HeLa cultures, but this time yeast extract was added to a final concentration of 4 % in broth. Growth was harvested after 3 days, since the tissue cells were by then almost completely destroyed by the high concentration of the yeast extract. Rod forms, as well as the usual granular and pleomorphic forms, could be observed in the cytoplasm of Giemsa-stained tissue cells, but agar subcultures yielded only large L form colonies. Bacillary colonies were not obtained at this point. A fourth serial passage of the harvest fluids was made in HeLa cells, this time using only 0.5 % yeast extract in broth. Pl. 1, fig. 5, illustrates the appearance of some of the organisms in these HeLa cells 3 days after inoculation. In this Giemsa-stained preparation, lying at the edge of the destroyed tissue cells, is a cluster of large L bodies, small granular forms, and long filamentous forms in which the purple rod forms were taking shape. The rod forms could easily be distinguished in the stained preparation by their colour and shape, but are difficult to identify in the photograph. Fluids from these Hela cultures were harvested on the third day and cultured on rabbit blood agar plates which were incubated in a candle jar. Duplicate cultures were made on ascitic fluid + veal infusion agar plates. After 5 days L colonies had appeared over the whole inoculated surface of the blood agar plates. In addition, there were one or two small corynebacterium colonies on each of these blood agar plates. To encourage if possible a conversion of the L form to a corynebacterium form, sterile hog gastric mucin, which has a growth-promoting effect on corynebacteria (Maccabe & King, 1951), was spread carefully over a part of the surface of the grown plates where only the L form colonies were present. A wide area around the corynebacterium colonies was left undisturbed. After re-incubation for another 2 days, the areas coated with mucin yielded pure cultures of corynebacterium colonies, whereas the areas without mucin showed no further development of corynebacterium colonies nor any change in the L colonies. The ascitic fluid plates which received no mucin yielded only a mixture of PPLO type and L type colonies. Pl. 1, fig. 6, illustrates the mixture of L and bacillary colonies which formed along the edge of the mucin-coated areas on the blood agar plates. The L colonies, composed of scattered granules and pleomorphic elements, were lying deeper in the agar than the dark bacillary colonies. The white forms in the bacillary colonies are rods which are lying above the level of focus. On the basis of the observed morphological changes, it appeared that conversion from PPLO through L form to a corynebacterium had taken place, but further evidence was needed to rule out the possibility of chance contamination having occurred.

Biochemical and serological studies of the derived corynebacterium strains

The two corynebacterium strains derived from PPLO on the blood agar plates, one in the presence of mucin, the other in the absence of mucin, were compared biochemically and serologically to determine their relationship to

the corynebacterium strain originally isolated from the urethral exudate. Culturally and biochemically these three strains were identical. All three fermented glucose but not maltose or sucrose, were catalase-positive, reduced nitrate to nitrite, did not hydrolyse urea or produce change in litmus milk. They produced small, black, shiny, raised, smooth colonies on tellurite agar.

Serologically the original strain and the two derived strains of corynebacterium were also identical. All three were agglutinated by a 1/1280 dilution of rabbit antiserum prepared against the original *Corynebacterium* sp. at the time of its isolation from the patient (Table 2). Moreover, the PPLO strain

Table 2. *Serological reactions of corynebacterium strains recovered, respectively, from initial urethral culture and from PPLO culture following tissue culture passage*

Strains	Agglutination titre of rabbit serum*	
	Anti-corynebacterium	Anti-PPLO
Original <i>Corynebacterium</i> sp.	1/1280	1/20
Corynebacterium derived from PPLO grown in tissue culture and subcultured on blood agar	1/1280	1/20
Corynebacterium derived from PPLO grown in tissue culture and subcultured on blood agar with mucin	1/1280	1/20
Original PPLO	1/640	1/1280

* Antisera were prepared against original strains of the organisms shortly after their isolation from clinical material.

was agglutinated by a 1/640 dilution of the anti-corynebacterium serum. Rabbit antiserum prepared against the original PPLO strain was also tested against the original and the two derived strains of corynebacterium. In each case agglutination occurred at a dilution of 1/20, whereas the PPLO strain was agglutinated by its homologous antiserum at a dilution of 1/1280. The growth inhibition test described by Edward & Fitzgerald (1954) was also employed to obtain additional evidence of antigenic relationship between the several growth forms, PPLO, L and bacillus. Anti-corynebacterium serum and anti-PPLO serum both inhibited growth of the PPLO form and the L form completely at 1/500 and partially at 1/1000. Several pools of normal rabbit serum used as controls did not cause any inhibition of growth of PPLO or L forms. These experiments showed that a definite antigenic relationship existed between the PPLO and the corynebacterium isolated from the original specimen, and strongly suggested the corynebacterium was actually derived from the PPLO.

DISCUSSION

The PPLO strain originally recovered from the urethral exudate and used in this study appeared to be a 'true' PPLO and not an L form; colonial appearance and morphological features were typical of PPLO. Early subcultures of the organism were established with ease in contrast to the difficulty of establishing

L phase cultures in stable form. All colonies appeared upon original isolation in equal distribution both near to and distant from penicillin in a cup on the agar. The strain was in no instance exposed to penicillin or other inhibitory agents after its first appearance on the original plates. Furthermore, this strain of PPLO, which has been carried for about one year in continuous stock passage on agar with frequent transfers to broth, has shown no tendency to revert to a bacillary form or even to that L stage which consists primarily of large bodies.

The corynebacterium strain isolated in a different section of the laboratory from a duplicate original specimen and kept completely separate from the PPLO cultures gave no evidence of being in the more or less labile L stage. In fact efforts to produce PPLO and L form colonies from the corynebacterium by cultivation on agar with penicillin, glycine, salts or by other methods failed to induce this conversion. Only in tissue culture was evidence obtained that this corynebacterium strain was capable of undergoing conversion at least to the L stage.

The experiments reported here have been carried out in detail only on the organisms isolated from a single case of non-specific urethritis. Work now in progress indicates, however, that the same relationship exists between PPLO and *Corynebacterium* sp. isolated by us from other cases of non-specific urethritis. A relationship between PPLO and the genus *Corynebacterium* has also been reported by other investigators. Minck (1953) showed that a number of so-called PPLO strains from the female genital tract were actually the L forms of corynebacteria. Peoples, Smith & Morton (1955) reported on the association of diphtheroids with the 'Campo' strain of PPLO in broth cultures. The question, therefore, arises: are the numerous strains of so-called PPLO isolated so commonly from the mucous membranes of human beings and animals in fact L forms whose bacterial parentage could be demonstrated if adequate efforts were made to reveal the relationship?

There is indeed an increased awareness of the possibility of confusing L forms with 'true PPLO'. Recent reviews by Edward (1954) and Klieneberger-Nobel (1954) and earlier reviews by Dienes & Weinberger (1951) and Tulasne (1951) deal at length with the similarities of and the distinctions between PPLO and L forms. Colonial appearance, cultural characteristics, morphological features, growth requirements, and the like are undoubtedly useful at times in attempting distinction between growth in the PPLO phase and growth in the L phase, but there is at other times sufficient overlapping of characteristics for these criteria to become wholly inadequate for classification purposes. Serological and metabolic properties are at present frequently disregarded when relationship to a parent bacterial form is unknown or unsuspected. The discussion of Dienes & Weinberger (1951) is pertinent in this respect and is here quoted in full: 'Organisms of the pleuropneumonia group usually can be isolated from the human throat and from the female genitals. These organisms were studied by several authors and their classification with the pleuropneumonia group is not questioned. No connexion of these organisms to bacteria is apparent. The reviewers believe that if the

3A L forms of *Salmonella* which do not return to bacillary forms were cultivated from similar sources, they would be regarded also as members of the pleuropneumonia group. There is nothing in the appearance of the colonies or in the morphology of the organisms to differentiate them from this group. Only the study of the serological and metabolic properties would identify them as *Salmonella* L cultures.⁷

It is our opinion that ordinary bacterial strains can exhibit all the characteristics of the PPLO form of growth as well as those of the L form, and furthermore, that the L form probably represents a transitional phase between bacillary and PPLO growth forms. Whereas an organism can be identified as the L form of a bacterium on the basis of serology, metabolism, or demonstration of actual conversion, there are no adequate criteria as yet for positive identification of an organism as a PPLO. Although a strain appears to be a PPLO because its growth form is 'fixed' and stable on artificial media, one should not assume that this will necessarily be so *in vivo*. From our observations, it seems likely that factors which produce changes in the host cells may in turn result in changes in the form or in the type of growth of the infecting organism, yet this may not always be readily apparent when the organism is again cultured *in vitro*. In the absence of actual demonstration of conversion from one to another growth form, the too rigid use of metabolic activities, growth requirements, perhaps even antigenic components as criteria of genetic relationship between growth forms so unlike as PPLO and bacilli may at times be misleading. For instance, the PPLO strain used in this study did not ferment glucose or any sugars tested, whereas the *Corynebacterium* spp. derived from the PPLO did ferment glucose.

The possible pathogenicity of the *Corynebacterium* sp. in its PPLO growth form or otherwise merits further study. There was clear-cut experimental evidence that in the presence of yeast extract or *Staphylococcus pyogenes* filtrate, the PPLO form of the corynebacterium was capable of multiplying rapidly in HeLa cultures and of producing extensive damage to the cells. It remains to be investigated whether or not the organism is capable of exhibiting toxigenic properties during some stage of its conversion from one growth form to another which might account for this cytopathogenic action. The experiments here reported do, in any case, suggest a promising line of approach for studies on the nature and pathogenicity of other organisms of the ill-defined pleuropneumonia group.

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EXPLANATION OF PLATE 1

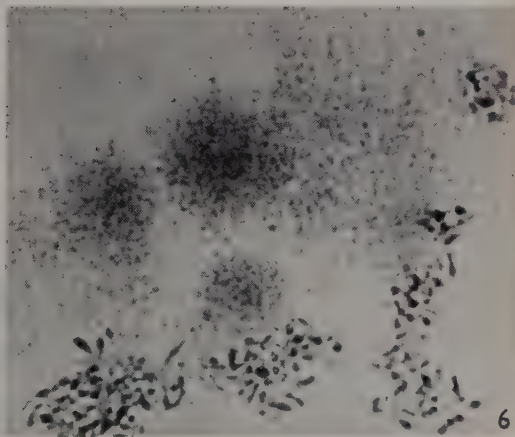
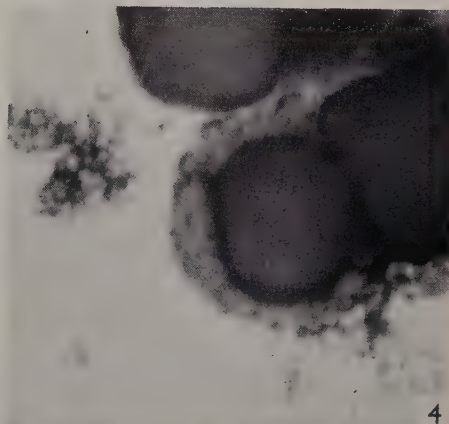
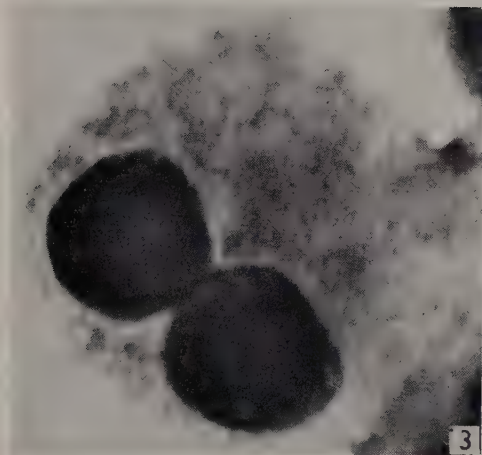
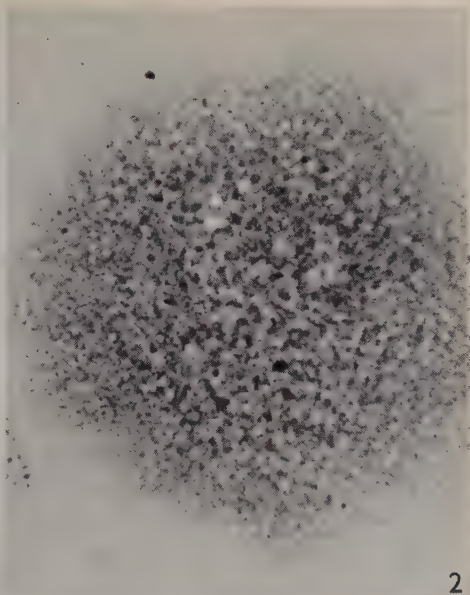
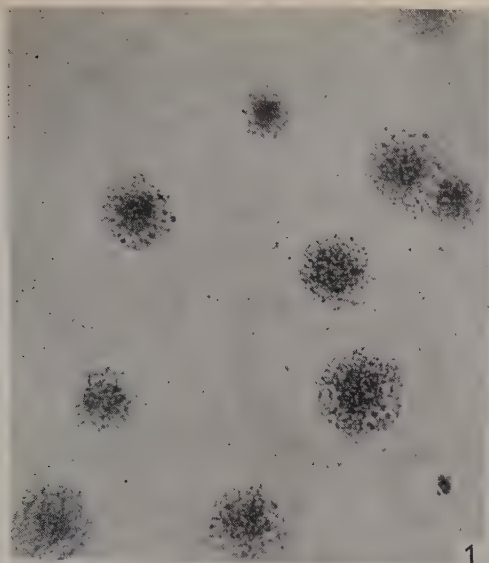
- Fig. 1. Seventy-two-hour culture of stock transfer of PPLO strain on ascitic fluid + veal infusion agar. Dienes's stain and phase contrast; $\times 360$.
- Fig. 2. Seventy-two-hour growth of the L form of a *Corynebacterium* sp. on ascitic fluid + veal infusion agar. Dienes's stain and phase contrast; $\times 900$.

Figs. 3, 4. HeLa cells photographed 72 hr. after inoculation with PPLO and yeast extract. The PPLO forms show as grey or black granules lying both intra- and extra-cytoplasmically. A large cluster of pleomorphic elements composed of L bodies and rod-shaped forms in a mass of amorphous material is lying free of the cells. Giemsa stain; $\times 1350$.

Fig. 5. HeLa cells photographed 72 hr. after inoculation with fourth serial passage PPLO and yeast extract. Large L bodies, small granular forms, and long filamentous forms are seen lying at the edge of the destroyed HeLa cells. The developing rod forms show as slightly darker and thicker portions of the filamentous forms. Giemsa stain; $\times 2700$.

Fig. 6. Seven-day growth of L and bacillary colonies of *Corynebacterium* sp. on rabbit blood agar plate treated with mucin. The bacillary colonies are composed of the large very dark elongated forms and the L colonies of the grey scattered granular and diffuse elements. Dienes's stain and phase contrast; $\times 900$.

(Received 11 January 1956)



R. G. WITTLER, S. G. CARY AND R. B. LINDBERG—REVERSION OF PPLO TO CORYNEBACTERIUM, PLATE I

(Facing p. 774)

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Cultivation of Animal and Plant Cells. By P. R. WHITE. London: Thames and Hudson. 1955. 239 pp. Price 35s.

Proceedings of the VIth International Congress for Microbiology. Rome, September 1953

Vol. 1. *General Microbiology.* 905 pp.

Vol. 2. *Immunology and Immunochemistry.* 409 pp.

Vol. 3. *Animal Viruses.* 509 pp.

Vol. 4. *Rickettsiae.* 445 pp.

Vol. 5. *Spirochetes.* 578 pp.

Vol. 6. *Microbiology applied to Human and Experimental Pathology.* 385 pp.

Vol. 7. *Industrial Microbiology and Microbiology of Fermentations.* 412 pp.
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'Surgery of the Heart and Thoracic Blood Vessels'. *British Medical Bulletin.* London: The British Council. 1955, 11, no. 3, 71 pp. Price 15s.

Teoría Citomórfica de la Hemopoyesis. By S. H. WAJDA. Scientific Report of the Institute of Histology and Embryology in Mendoza, R. Argentina. 1954. 204 pp.

[The Editors of the Journal of General Microbiology accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers are published as received from authors.]

THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Twenty-first General Meeting in the University of Sheffield on Tuesday and Wednesday, 13 and 14 September 1955. The following communications were made:

COMMUNICATIONS

The Occurrence of Motile Cells, which are not Mutants, in some *Salmonella* O Strains. By C. QUADLING and B. A. D. STOCKER (*The Lister Institute, London*)

When pour-plates of non-motile *Salmonella* strains in semi-solid agar are incubated, each viable cell produces a colony of approximately 2 mm. diameter; in 20 of 47 non-motile strains tested these colonies are surrounded by smaller 'satellite' colonies, which on subculture yield non-motile growth indistinguishable from the parent strain. Homologous anti-flagellar serum prevents satellite formation. Each satellite colony is inferred to develop from a spontaneously produced motile cell, which is not a mutant, since its progeny are non-motile.

The proportion of such motile cells has been estimated in *Salmonella typhimurium* O strain SW 545, of Stocker, Zinder & Lederberg (1953). Pour-plates in semi-solid agar, with 4% gelatine, were first incubated at 37°, chilled, and then incubated at bench temperature. Motility was prevented by the solidification of the medium, but growth continued until colonies (and satellites) were visible. After 5 hr. incubation at 37° about one-third of the colonies had no satellites. Assuming that 'events' leading to the production of satellites occurred at random amongst the population, the mean number of such events could be calculated from the Poisson distribution. From the number of viable cells per colony at the time of chilling, the event frequency per cell per bacterial generation was found to be about 10^{-5} . The distribution of numbers of satellites per colony indicated that an event resulted in an average of 2-3 satellites.

Motile cells were isolated from strain SW 545 by micro-manipulation. The majority of the progeny of such cells were non-motile; usually a few (maximum, four) motile cells were present, even after sixteen cell generations. They behaved as if their motility resulted from the presence of non-multiplying *motility-conferring particles*. An event probably consists of the transient ability, in a cell, to synthesize such particles.

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STOCKER, B. A. D., ZINDER, N. D. & LEDERBERG, J. (1953). *J. gen. Microbiol.* **9**, 410.

Synergism between Sulphamezathine and Daraprim in the Treatment of Toxoplasmosis in Mice. By J. K. A. BEVERLEY and B. A. FRY (*Department of Bacteriology and Department of Microbiology, University of Sheffield*)

Mice were infected intra-nasally with 15,000 toxoplasma (750 LD₅₀) and then given a standard diet (diet 86) containing various fractions of the toxic doses of one or more drugs. The toxic doses (i.e. doses preventing 6- to 8-week-old mice from gaining weight) were respectively 1.6 g. sulphamezathine, 200 mg. 4:4'-diaminodiphenylsulphone (DDS), and 50 mg. daraprim per 100 g. of food.

Even with half of the toxic dose of daraprim, DDS or sulphamezathine, relapses occurred after treatment (28 days) was stopped, and of the mice surviving a further 28 days on the normal diet, some were still carriers of toxoplasma. Sulphamezathine and DDS together gave no greater protection than either alone. With daraprim plus either sulphamezathine or DDS, a synergistic effect was apparent. Thus with one-eighth of a toxic dose of daraprim plus one-eighth of a toxic dose of DDS or sulphamezathine, all the mice survived 28 days on the normal diet; 95% (daraprim + DDS) and 100% (daraprim and sulphamezathine) of the mice were free from toxoplasma. The synergism may be explained on the grounds that DDS and sulphamezathine interfere with the synthesis of 'folic acid', whilst daraprim inhibits one or more of the biosyntheses in which folic acid is involved. The 'pseudocysts' of toxoplasma may be more susceptible to daraprim than to sulphamezathine, and actively growing parasites may be eliminated only by concentrations of daraprim which are also toxic to the host. If this were so, then conditions can be envisaged in which synergism between sulphamezathine and daraprim would be possible. Eyles & Coleman (1953) have reported synergism between sulphadiazine and daraprim and obtained cures in up to 75% of infected mice.

REFERENCE

EYLES, D. E. & COLEMAN, N. (1953). *Antibiotics and Chemotherapy*, **3**, 483.

Reversed Allergy in Acute Infections. By J. K. A. BEVERLEY (*Department of Bacteriology, University of Sheffield*)

Daily measurements of the extent of toxoplasmic lesions in rabbits, treated with 4:4'-diaminodiphenylsulphone (DDS), showed that in non-immune animals there were two periods of more intense inflammation separated by a period of partial remission. The second phase started between 5½ and 8 days. It was more intense the less the delay in its onset. It was abolished by giving cortisone as well as DDS, even though cortisone, when given alone, had no effect on the lesions. A rabbit which had no γ -globulin was the only one which did not develop a secondary reaction.

It was suggested that the primary reaction is towards a toxic stimulus, and that the second phase is a reaction between residual antigen and newly formed antibody. In self-resolving lesions the biphasic nature of the reactions is not

appreciated because it is not until the development of an immune mechanism commences that the stimulus of the primary toxic response is curtailed. Consequently, the two reactions merge into one another. It is only when the primary phase is curtailed that an interval between the two reactions occurs.

The lesions produced in immune rabbits developed much more quickly, were more intense and were monophasic. The difference from the response in non-immune animals was explained by showing that the non-immune response is a sequence of two types of reactions (a toxic and an allergic), whereas the response in immune animals is a summation of the same two reactions occurring concurrently.

Atypical Anaerobic Forms of *Streptomyces pyogenes* Associated with Resistance to the Tetracycline Antibiotics. By E. J. L. LOWBURY and L. HURST (*Medical Research Council Industrial Medicine and Burns Research Unit, Birmingham*)

Tetracycline resistant *Streptomyces pyogenes* were isolated from the burns of a number of patients, and most of them showed the following characteristics: (1) typical anaerobic growth in nutrient broth and on ordinary blood or serum agar; (2) no aerobic growth on these media; (3) aerobic growth in a small colony form on concentrated agar media containing blood or serum; (4) sensitivity to penicillin and erythromycin.

After storage or subculture these 'AN' forms tended to revert to aerobiosis on ordinary media, but remained resistant to the tetracyclines.

Most of the 'AN' forms were agglutinated by sera of types 5, 27 and 44, but two other types (2 and 4) were found. More than half of the patients showing these atypical streptococci had not been treated with tetracyclines.

'AN' forms were found to grow under conditions in which hydrogen peroxide production from typical strains of *Streptomyces pyogenes* could not be demonstrated.

The clinical importance of these organisms is apparent in their association with skin graft failure and their resistance to tetracyclines. As systemic penicillin will not clear burns of *Streptomyces pyogenes*, erythromycin therapy is indicated for infections with tetracycline resistant streptococci.

Penicillin-destroying Variants from Penicillin-sensitive Staphylococci.

By MARY BARBER (*St Thomas's Hospital Medical School, London*)

When penicillin-sensitive strains of staphylococci are cultivated in the presence of penicillin *in vitro* many types of penicillin-resistant variant appear, but most workers agree that variants isolated in this way do not destroy penicillin. Recently, however, two workers (Szybalski, 1953; Gould, 1955) have claimed the isolation *in vitro* of penicillinase-producing staphylococci from penicillin-sensitive strains. Neither paper gives details or states what precautions were taken to avoid contaminants, but apparently the variants resembled the type of strain isolated from patients with penicillin-resistant staphylococcal infection.

In experiments over a period of 6 years involving 26 penicillin-sensitive strains of *Staphylococcus aureus*, the present author has failed to isolate such variants. Strains with very weak penicillin-destroying activity have, however, been isolated fairly frequently.

When a penicillin-sensitive strain of *Staphylococcus aureus* is plated out on to a penicillin ditch plate, after 24 hr. incubation there is a fairly straight line of inhibition. After 4–7 days' incubation, however, many colonies appear in the area of inhibition. By repeated subculture from such colonies two strains (a penicillin-sensitive variant of a penicillinase-producing organism, D3S, and the Oxford *Staphylococcus*) have been repeatedly shown to yield variants which destroy small quantities of penicillin very slowly. Thus each of 6 such isolated from the Oxford *Staphylococcus* inactivated 1 unit of penicillin in 3 hr., and 6 isolated from D3S took several days to inactivate the same amount of penicillin.

The penicillin-destroying variants isolated from the Oxford *Staphylococcus* showed weak or absent coagulase activity and were not typable by the bacteriophage method. Those isolated from D3S were coagulase-negative, but those tested still showed phage reactions similar to those of the parent culture. Repeated subculture in the presence of penicillin did not increase the penicillin-destroying activity.

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The Isolation of Proflavine Resistant Mutants of *Escherichia coli* by the Replica Plating Technique. By MARGARET J. THORNLEY and J. YUDKIN (*Queen Elizabeth College, University of London*)

Proflavine resistance develops gradually in cultures of *Escherichia coli* repeatedly grown in its presence, and therefore conforms to the 'penicillin pattern' of drug resistance. To account for this, Demerec suggested a series of step-wise mutations. This theory has been criticized on the grounds that the experiments involved direct contact between cells and drug, with the possibility of interaction. It is now possible, by the replica plating technique, to isolate resistant cells without their coming in contact with the drug. In the present work, a modification of this method has been used with mutants showing proflavine resistance.

By replica plating of the sensitive strain of *Escherichia coli* C3 a first-step mutant with a 19-fold increase in resistance was isolated. A second-step mutant was derived from the first in the same way, the resistance being enhanced about three times, and from this a third-step mutant with a 1.5 times higher resistance was obtained. This third-step mutant could withstand 85 times the concentration of proflavine tolerated by the parent sensitive strain, although it had never been exposed to the drug during the selection process.

It is clear that step-wise mutations do take place in the complete absence of the drug concerned, and that this process can account for the gradual acquisition of the 'penicillin type' of drug resistance.

Tetrazolium Media for the Isolation and Differentiation of Lancefield Group D Streptococci. By ELLA M. BARNES (*Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge, and Department of Scientific and Industrial Research*)

2:3:5-Triphenyl-tetrazolium chloride has been used in a nutrient glucose medium to show the considerable differences in reducing properties between some of the Lancefield group D streptococci. The ability to reduce tetrazolium to formazan, in glucose broth at an initial pH 6.0, distinguishes strains of *Streptococcus faecalis* and its haemolytic and proteolytic variants *zymogenes* and *liquefaciens* from *S. faecium* (Orla Jensen), *S. durans* and *S. bovis*. Measurements of the oxidation-reduction potentials in growing cultures show that *S. faecalis* grows at an E_h about 150 mV. lower than the other species. The differences in reducing properties between *S. faecalis* and *S. faecium* correspond with many other tests now used to separate these species which for many years were classified together. Confirmation of the differences in reducing properties was obtained by testing 68 freshly isolated strains of *S. faecium* and 34 strains of *S. faecalis*.

Thallos acetate (0.05 or 0.1%) may be incorporated in a tetrazolium glucose agar (pH 6.0) for the selective isolation of the streptococci. Colonies of *S. faecalis* and variants have deep red centres, whilst those of other group D streptococci are white.

Typing of Adenoidal-Pharyngeal-Conjunctival (APC) Viruses by Complement Fixation. By H. G. PEREIRA (*Common Cold Research Unit, Salisbury*)

APC viruses form a group of agents sharing certain properties and possessing common antigens demonstrable by complement fixation. Six types of viruses have so far been distinguished in this group, by neutralization tests in tissue cultures. Complement-fixation tests performed with sera from hyperimmunized rabbits reveal cross-reactions with all types, but higher titres are always obtained with the homologous pairs. Based on this fact, the complement-fixation test was applied to the typing of these viruses.

Sera from hyperimmunized rabbits were standardized by chess-board complement-fixation tests against the six type antigens and serum dilutions giving maximal type differentiation were selected. Typing was carried out by testing tissue culture antigens from new strains against standard dilutions of each of the six sera.

Ten strains, including types 1, 2, 3 and 5, were typed by neutralization tests in HeLa cell cultures and by complement fixation and the results of the two tests showed perfect agreement.

Some Factors Influencing the Viability of Freeze-Dried BCG. By J. P. FARMER, P. W. MUGGLETON and J. UNGAR (*Glaxo Laboratories Ltd., Greenford, Middx.*)

The advantages of presenting BCG vaccine in the freeze dried state are well recognized. The product must have a sufficient number of viable BCG cells and this viability must be retained on storage (e.g. for 12 months).

Previous investigations have shown that a satisfactory viability can be retained in a number of different media, but most of these have such defects as unsatisfactory appearance (e.g. concentrated sugar solutions) or unsuitability for injection (e.g. serum). In attempts to find a better medium, dextran, having molecular dimensions similar to those used for blood volume expanders, was found to have advantages. The addition of small amounts of glucose (5.0–7.5%) prevent dextran solutions from being overdried, a necessary precaution since suspensions of BCG in plain dextran solutions are easily dried to death. Larger amounts of glucose cause the retention of too much water with a consequent loss of viability on storage.

The BCG for this investigation was grown in deep culture in Sauton's medium with the addition of 1/4000 (w/v) of Triton W.R. 1339, which has the advantage over the traditional surface cultivation that the presence of clumps and milling of the growth are avoided. BCG cells grown in deep culture have a tendency to aggregate in dextran solutions; this is avoided by adding 1/4000 Triton W.R. 1339 to the drying medium.

On freeze drying BCG in dextran + glucose solution (or serum), there is a negligible loss of viability during the freezing (whether rapid or slow) and during the removal of the first 25–50% of the water. Thereafter there is a progressive diminution of viability as water is removed, this loss being unaffected by the speed at which the water is removed.

Evaporative freezing (in an Edward's centrifugal freeze drier) has been compared with freeze drying after pre-freezing to -60°C . The former method may give a better preservation of viable organisms.

Growth Curves of Strains of Pleuropneumonia-like Organisms. By M. BUTLER and B. C. J. G. KNIGHT (*Department of Microbiology, The University of Reading*)

The method of Miles & Misra (1938) was used to count the viable particles in static liquid cultures of pleuropneumonia-like organisms (PPLO; *Mycoplasma* spp.). The liquid medium for cultivation was the selective medium of Edward (1947). Drips (0.02 ml.) of diluted samples, taken at intervals during the period of incubation (37°), were placed (in triplicate) on the surface of plates of the same medium solidified with agar, in the usual way. The smallness of the typical colonies enabled as many as 300 colonies/drop area to be counted. Two strains of *Mycoplasma* spp. were mainly used (bovine pleuropneumonia, bovine genital). Both organisms increased to 10^9 – 10^{10} viable particles/ml. in 36–48 hr., and the count then fell steeply. Any maximum stationary phase

must have been less than 12 hr. in duration. The samples were examined concurrently by phase-contrast microscopy. With the bovine genital strain short rod-like elements became visible in the microscope at *c.* 28 hr. (viable count: 10^7 – 10^8 particles/ml.); at 36 hr. most of the particles were in groups of 2–6 (count; 10^{10} /ml.). Visible units per group and viable count thereafter declined; highly refractile bodies (2 – 6μ .) appeared at 60 hr. At 5 days only a few granules, debris and the refractile bodies remained (viable count 10^5 – 10^6 particles/ml.). The observations were analogous with the pleuropneumonia strain except that the initial small ($0.8 \times 0.4\mu$.) granules (24 hr.) appeared to aggregate without visible connexion, grew larger and showed somewhat yeast-shaped bodies ($1 \times 2\mu$.) by 72 hr. These later disappeared.

One saprophytic strain (sewage A of Laidlaw & Elford) showed a lag and log phase similar to the other two organisms; but in contrast to these showed a long maximum stationary phase.

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MILES, A. A. & MISRA, S. S. (1938). *J. Hyg., Camb.* **38**, 732.

The Effect of Nucleic Acid Fragments on the Growth of a Pleuropneumonia-like Organism. By S. CROWTHER and B. C. J. C. KNIGHT (*Department of Microbiology, University of Reading*)

Edward (1952, 1954) observed that certain strains of pleuropneumonia-like organisms (PPLO; *Mycoplasma* spp.) from bovine genital tracts would not grow on his selective medium, unless certain material, e.g. vaginal mucus, hog gastric mucin, was added to the basal medium. He showed that these growth-promoting materials could be replaced by thymus deoxyribonucleic acid (DNA), but not by the deoxyribosides of thymine and guanine. We have further studied this observation and find that the growth-promoting effect of DNA occurs because it annuls an inhibitory effect of yeast extract (Oxoid), which was a component of the basal medium. This antagonistic effect can be reproduced with DNA versus yeast ribonucleic acid (RNA), when the normal content of yeast extract in the medium is decreased. (The yeast extract cannot at present be eliminated completely because it may contain a factor needed with some batches of horse serum, which is also a component of the medium.)

Both DNA (thymus) and RNA (yeast) have now been partially hydrolysed by various methods (chemical, enzymic), the products partially fractionated and then tested for the antagonistic effect. The mononucleotides and smaller fragments are inactive; the active fragments must be larger than mononucleotides.

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Germination and Biochemical Activity of Spore Suspensions of some Aerobic Spore Formers at Low Temperature. By S. A. Z. MAHMOUD and J. WOLF (*Department of Agriculture, University of Leeds*)

Purified spores of *Bacillus cereus* and *B. subtilis* were suspended in various nutrient broths, soil extracts, autoclaved soil of various moisture contents, and three inorganic menstrua: phosphate pH 7.2, Ringer's and distilled water, and incubated at 8°, 5°, 1° and -2° for periods up to 270 days. Periodic total and spore counts on plates indicated a progressive decrease in each count caused by germination taking place in all except the three inorganic media. Chemical tests for enzyme secretion and activity indicated the presence of gelatinase, nitrataase and, with spores of *B. pasteurii*, urease. Enzyme activity was correlated with germination, and is attributed to enzyme secretion as a result of germination.

Bacillus subtilis germinated to a greater extent than *B. cereus* in each of the nutrient media, although germination was rarely complete even with the former. Similar germination was observed with these two organisms in L- and D-alanine at low temperature; in the latter it is probably a result of racemization to the L-form.

Examination of data in the literature suggests that similar germination occurs with spores of anaerobes, even at higher temperatures under conditions unfavourable for vegetative growth, and raises the possibility of toxin production and nitrogen fixation by the non-proliferating but germinating spore.

Effects of L- and D-Alanine on the Germination of some Aerobic Spore Formers. By J. WOLF and S. A. Z. MAHMOUD (*Department of Agriculture, University of Leeds*)

The effects of various concentrations of L- and D-alanine in phosphate at pH 7.2 were studied with suspensions of *Bacillus cereus* and *B. subtilis* at 30° over several days. 60% germination was obtained with *B. cereus* in various concentrations of L-alanine. In D- inhibition was complete at 1 mg./ml. but not at 0.1 g. With *B. subtilis*, however, germination, growth and resporulation were obtained in L- and D-alanine, the different behaviour of the two species being correlated with their different N requirements as shown by Knight & Proom in 1950. Reversibility of alanine racemase was established by the use of kidney D-amino-oxidase and L-amino-oxidase from *Neurospora*. The *Q* values for the L → D reaction were higher than in the reverse direction and the suggestion is made that the lower value is due to enzyme derived from spore coat only, whereas in the L → D reaction there is additional enzyme derived from internal cell, made available by germination.

Neither L- or D-alanine were found to affect death or sporulation rate of vegetative cells of *Bacillus cereus*. A survey of germination requirements of several species suggests a broad correlation with nutritional (N) requirements.

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Metabolism of Phosphate Esters by Extracts of *Thiobacillus denitrificans*.

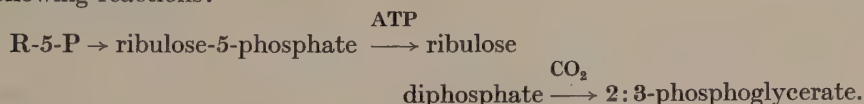
By P. A. TRUDINGER (*Agricultural Research Council Unit of Microbiology and Department of Microbiology, the University, Sheffield*)

Cell-free extracts of the chemosynthetic autotroph, *Thiobacillus denitrificans*, prepared either by extraction of freeze-dried bacteria or by crushing fresh cells in the Hughes press, were found to contain the following enzymes (cofactor requirements are shown in parentheses): glucose-6-phosphate dehydrogenase (TPN, Mg^{2+}), 6-phosphogluconate dehydrogenase (TPN), enolase (Mg^{2+}), aldolase (Co^{2+}), hexose monophosphate isomerase, hexose diphosphatase (Co^{2+} , Mn^{2+} , Fe^{2+} or Zn^{2+}) and pentose phosphate isomerase. The presence of transketolase and transaldolase was indicated by the rapid conversion of ribose-5-phosphate (R-5-P) to hexose monophosphate and what appeared to be tetrose phosphate.

Crude, dialysed extracts fixed $^{14}CO_2$ in the presence of Mg^{2+} and ATP. Fixation was increased two or three times by the addition of R-5-P, the increase being ATP dependent.

Among the end products of $^{14}CO_2$ fixation was a compound which behaved on paper chromatograms as 3-phosphoglycerate. On hydrolysis of this compound with phosphatase, a substance was produced which could not be distinguished from glyceric acid by paper chromatography. Periodate oxidation showed that all the ^{14}C in the glyceric acid was in the carboxyl group. Addition of R-5-P increased sevenfold the amount of labelled phosphoglycerate produced during $^{14}CO_2$ fixation. 2-Phosphoglycerate and phospho-enolpyruvate were also shown to be products of the fixation of $^{14}CO_2$ in the presence of R-5-P.

The fraction of the crude extract which precipitated at 40–50% saturation of ammonium sulphate, contained an enzyme system which fixed $^{14}CO_2$ only in the presence of both R-5-P and ATP. 93% of the ^{14}C fixed appeared in phosphoglycerate. It is suggested that the fixation was the result of the following reactions:



Some Aspects of the Photometabolism of L-Glutamic Acid by *Rhodospirillum rubrum*. By G. S. COLEMAN (*Department of Biochemistry, University of Cambridge*)

The photometabolism of L-glutamic acid by washed suspensions of a mutant strain of *Rhodospirillum rubrum* S1 has been studied manometrically. This strain, unlike the parent, produced hydrogen in the presence of L-glutamic acid when the gas phase was argon and also had an appreciable endogenous hydrogen and carbon dioxide production. The presence of a caustic potash (KOH) paper markedly affected the course of the metabolism and halved the rate of glutamic acid disappearance from the supernatant. In the presence of a KOH paper the rate of hydrogen production was constant for 3 hr. but in its

absence the production of hydrogen and carbon dioxide reached a maximum after 90 min. This was associated with the appearance of ammonia in the medium and was followed by a gas uptake which probably represents a photo-reduction of the carbon dioxide by the hydrogen. During the first 90 min. of the reaction more hydrogen and carbon dioxide were produced per mole of glutamic acid metabolized than when a KOH paper was absent. Under all the conditions studied, of the glutamic acid which disappeared, 90% of the carbon and nitrogen was converted to intracellular products.

In the presence of gaseous nitrogen, hydrogen production was eliminated and the carbon dioxide formation reduced. The metabolism of glutamic acid under hydrogen was greatly complicated by the uptake of hydrogen and carbon dioxide by the cells. Despite this there was a net output of alkali insoluble gas in the presence of substrate which indicated that hydrogen production from glutamic acid is not inhibited under these conditions.

The Effect of Arsenite on the Metabolism of *Rhodospirillum rubrum*.

By J. G. ORMERD and S. R. ELSDEN (*A.R.C. Unit of Microbiology, Department of Microbiology, Sheffield University*)

Arsenite (10^{-3}M) inhibits (c. 80%) the photometabolism of acetate and pyruvate by *Rhodospirillum rubrum* but has a smaller effect (c. 20%) on the photometabolism of succinate, fumarate, malate and butyrate. In contrast the dark oxidation of all substrates is strongly inhibited (c. 75%) by arsenite. Fluoroacetate behaves similarly to arsenite. In the light, fluoroacetate inhibition is accompanied by the accumulation of citrate in the presence of acetate, pyruvate and oxaloacetate; and in the dark large amounts of citrate are accumulated in the presence of the 4-C dicarboxylic acids.

We have interpreted the results with fluoroacetate as follows. Substrates such as succinate, the photometabolism of which is only slightly inhibited, and from which no citrate is produced, are metabolized by a pathway which does not involve the tricarboxylic acid cycle (TAC). Arsenite is known to inhibit the oxidation of keto acids and, since it inhibits the photometabolism of pyruvate but not of succinate, we conclude that succinate is not metabolized via free pyruvate. The possibility exists that succinate is assimilated via phosphopyruvate.

The fluoroacetate inhibition of the dark oxidation of succinate with the formation of citrate suggests that in this case a more extensive degradation of the substrate is involved and that the TAC is essential. Keto acids accumulate when succinate is oxidized in the dark in the presence of arsenite and α -ketoglutarate accounts for 90% of the total keto acids. Approximately 0.5 mole α -ketoglutarate is formed/mole of succinate utilized. These facts support the view that the dark oxidation of succinate involves the TAC.

Some Factors affecting Induced Enzyme Formation in Bacteria. By H. TRISTRAM (*University College, London*)

Enzyme Formation and Nucleic Acid Synthesis in *Staphylococcus aureus*.

By E. H. CREASER (*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

It has been shown that washed suspensions of *Staphylococcus aureus* will synthesize the enzymes catalase, β -galactosidase and glucozymase when supplied with a mixture of amino acids, glucose and galactose. Previous work with the purine analogue 8-azaguanine indicated that whereas the formation of catalase and β -galactosidase was dependent upon the synthesis of ribonucleic acid (RNA), glucozymase formation was not. The present work was undertaken in order to confirm this finding by the direct measurement of the synthesis of ribo- and deoxyribonucleic acid (DNA).

It was found that washed suspensions of *Staphylococcus aureus* would incorporate ^{14}C labelled uracil into the pyrimidines of cellular nucleic acid, such incorporation being linear with time and not significantly reversible. Incorporation of radioactivity from ^{14}C uracil was used as a measure of the synthesis of DNA and RNA.

When RNA synthesis was stimulated by the addition of purines to the system described above there was an increased formation of catalase and β -galactosidase, but not of either glucozymase or DNA. Similarly, glucose, in the presence of purines, stimulated the synthesis of RNA, catalase and β -galactosidase but not of DNA or glucozymase.

The effect of several antibiotics upon the syntheses of enzymes and nucleic acid was investigated and it was found that when RNA synthesis was inhibited there was a decrease in enzyme formation; antibiotics which inhibited enzyme formation also reduced the synthesis of DNA. If amino acids were omitted from the system there was no significant enzyme formation and RNA synthesis was decreased.

Enzyme formation could be inhibited by exposing the cells to X-irradiation, the results obtained indicating that the enzyme-forming system had a target size of approximately 60 m μ .

The Urease Activity of *Corynebacterium renale*. By A. J. LISTER (*Department of Biochemistry, University of Cambridge*)

Corynebacterium renale is the causative organism of bovine pyelonephritis. The bacterium possesses a constitutive urease which produces 25,000 μg . of ammonia/mg. dry weight of cells/hr. A study has been made of the metabolism of the organism to determine whether this high urease activity plays an essential role in the pathogenesis of pyelonephritis. A cell-free extract of the enzyme has been prepared with the following properties: a Michaelis constant of 0.030 M, an optimum pH value of 7.5, and a temperature velocity constant between 15° and 45° of 7800 cal. Urea is broken down stoichiometrically producing 2 molecules of ammonia/molecule of urea.

Experiments using urea or ammonium chloride labelled with ^{15}N have shown that during growth in a 1% peptone broth containing 1% glucose, up to 40%

of the nitrogen assimilated by the organism can be derived from added urea or ammonia. No defined synthetic medium is yet available for a more detailed study of the role of urea and urease in the nutrition of *Corynebacterium renale*. Growth can be obtained on media containing an acid hydrolysate of casein, but not on media in which the casein hydrolysate has been replaced by an equivalent mixture of synthetic amino acids.

Three mutant strains of *Corynebacterium renale* which are lacking in urease activity have been isolated from X-irradiated cultures, and are being tested for pathogenicity towards mice.

Nuclear Morphology of *Bacillus cereus* grown on a Defined Medium. By MARION FAIRMAN (*University of Glasgow*)

When *Bacillus cereus* was grown on the surface of agar plates containing a defined basal* medium plus 1 % glucose and 1 % urea, the organism developed well-marked lipid inclusions. These inclusions were not seen if the organism was cultured on a full medium such as meat-extract agar. The lipid nature of the inclusions was established by sudan staining methods. Phase-contrast microscopy showed dark granules corresponding to the lipid inclusions in distribution, shape and size. When the organisms were stained for nuclear material by the osmic acid-hydrochloric acid-Giemsa method, the lipid remained unstained but was seen as refractile inclusions. These inclusions were very closely associated with the nuclear material, often appearing to be surrounded by an unbroken ring, or a granular band, or a horse-shoe shape of nuclear material. These observations support the suggestion of Delaporte (1950) that lipid inclusions may distort nuclear shapes. It is possible that distortions of the kind observed could be misinterpreted as mitotic figures.

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Growth and Division of Protoplasts of *Bacillus megaterium*. By K. MCQUILLEN (*Department of Biochemistry, University of Cambridge*)

Protoplasts formed by lysozyme dissolution of the cell walls of *Bacillus megaterium* have previously been shown to be capable of synthesizing proteins, nucleic acids, enzymes and of supporting the growth of bacteriophages. It has now been found that incubation in the presence of glucose and amino acids leads to increases in size and dry weight of protoplasts—e.g. doubling of the dry weight after 2 hr. at 28°. After 6–8 hr., some of the enlarged protoplasts had developed a small protuberance; this later became enlarged and ultimately dumbbell-shaped forms were observed.

Phase-contrast photomicrographs and electron micrographs illustrate the sequence of forms during the growth and division of protoplasts.

* Composition of basal medium: dipotassium hydrogen phosphate (K_2HPO_4) 1 g., sodium chloride 2 g., magnesium sulphate 0.5 g., ferric chloride, trace, calcium carbonate 10 g., tap water 1000 ml.

Some Observations on the Breakdown of Pyruvate by Cell-free Extracts of a Rumen Micro-organism (LC1). BY J. L. PEEL (*A.R.C. Unit for Microbiology, The University, Sheffield*)

In presence of N_2 , extracts of vacuum-dried cells of the rumen organism LC1 catalyse a rapid release of gas from pyruvate, which is phosphate dependent. The overall reaction approximates to the following equation and resembles that already known to occur in *Escherichia coli* and in *Clostridium butylicum* and *C. butyricum*:



Arsenite at a concentration of $10^{-5}M$ has no effect on the reaction as measured by H_2 production, and no gas is produced from formate. In these respects, the system is similar to that of *C. butyricum* and distinct from that of *Escherichia coli*.

Whole extracts also catalyse the transfer of hydrogen from pyruvate to 2:6-dichlorophenolindophenol, but a fraction prepared by precipitation with ammonium sulphate is only active in this test when fortified with an extract of boiled, dried cells. So far it has not been possible to replace the boiled extract by mixtures of known co-factors of pyruvate oxidation, including coenzyme A, diphosphothiamine and Mg^{++} , the cofactors required by *Clostridium butyricum*.

The system also differs from that of *Clostridium butyricum* in another respect. The whole extract of LC1 catalyses a slow breakdown of acetyl phosphate which is accelerated by arsenate, suggesting the presence of phosphotransacetylase. However, the arsenolysis is not stimulated by the addition of extra coenzyme A to the system, and furthermore, the phosphotransacetylase activity as measured by the arsenolysis reaction is very low compared with the rate at which pyruvate is broken down according to the above equation. It is concluded that a phosphotransacetylase of the type present in *C. kluyveri* cannot be involved in pyruvate breakdown. The pyruvate-oxidizing system of this organism thus appears to be different from those already studied in other organisms.

Some Properties of Mutants of *Escherichia coli* requiring Citrulline and a Pyrimidine for Growth. BY J. C. HOLDAWAY (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

The work reported by Back & Woods (1953) with a mutant (A) requiring for growth citrulline plus either uracil, orotic acid or carbamylaspartic acid has been extended with this mutant and with two others responding to citrulline plus uracil or orotic acid (B) and to citrulline plus uracil (C). In each case the mutation is probably at a single locus since reversions have always been to prototroph characters.

Cell suspensions of the parent strain and mutant A convert carbamylaspartate to orotate plus uracil, and orotate to uracil; uracil is only formed

if glucose is present. Cells of mutant C cannot metabolize orotate and convert carbamylaspartate only to orotate.

Mutant A grows on carbamylaspartate and orotate with increased lag compared with uracil; this is abolished after a single subculture. Cells so adapted to carbamylaspartate are not adapted to orotate, but cells adapted to orotate are adapted to carbamylaspartate (Back & Woods, 1953 and unpublished observations). When the glucose of the medium was replaced by lactate, growth on orotate and uracil was indistinguishable, and showed only a slightly increased lag on carbamylaspartate; this lag was not reduced on subculture.

No evidence could be found (Back, unpublished observations) to support a previous hypothesis that the growth requirements might result from an accumulation of carbamylglutamate. The possibility that the lesion was a failure to synthesize or transfer a carbamyl group was examined. Carbamylphosphate (Jones, Spector & Lipmann, 1955; Grisolia, Wallach & Grady, 1955; Marshall, Hall & Cohen, 1955) did not replace citrulline plus pyrimidine for growth. In cell-free preparations mutant A had 80% of the activity of the parent strain in transferring the carbamyl group of carbamylphosphate to ornithine and aspartate. Such preparations had so little ability to synthesize carbamylphosphate (from NH_3 , CO_2 and adenosinetriphosphate) that no valid comparison between mutant and parent strain was possible.

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Some Properties of a Mutant of *Escherichia coli* requiring Vitamin B₆ for Growth. By J. G. MORRIS and D. D. WOODS (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

This mutant (no. 166, obtained from Dr B. D. Davis) grows on a glucose-ammonium salts medium supplemented with either pyridoxin (10^{-6}M) or a mixture of serine and glycine (each $5 \times 10^{-4}\text{M}$); either amino acid suffices, but a mixture gives more consistent results. When all three substances are present growth is as rapid as with the prototroph, but with either pyridoxin or serine-glycine mixture alone onset of growth is delayed about 24 hr. and the final cell density reduced by 20–30%.

Serine and glycine, however, will not replace pyridoxin with a medium containing lactate as carbon source or with a medium containing glucose sterilized by filtration or by autoclaving at a pH below 7. In a survey of possible products formed by autoclaving glucose at pH 7 to 8 only glycolaldehyde (5×10^{-5} to 10^{-3}M) permitted the organism to grow in the presence of serine-glycine and with no added pyridoxin. This occurred with either lactate or Seitz-filtered glucose as carbon source; with the latter and with 10^{-3}M -

glycolaldehyde present the delay in growth was abolished but the extent of growth was not enhanced.

Organisms grown on a medium containing serine, glycine and glycolaldehyde contained 0.1 μ moles B₆ (as pyridoxin)/g. dry wt. A similar figure was obtained for cells harvested from media containing pyridoxin or serine, glycine and pyridoxin. The present experiments do not permit a conclusion as to whether the mutant can synthesize vitamin B₆ if supplied with serine, glycine and glycolaldehyde, or whether these substances are products of the function of vitamin B₆ whose presence permits the organism to grow with smaller (and normally synthesized) amounts of the vitamin.

Nutritional Studies in *Vibrio cholerae*. By K. BHASKARAN (*Nuffield Foundation Fellow, Wright-Fleming Institute of Microbiology*)

One hundred and fifty-one *Vibrio cholerae* were investigated for their growth requirements. Seventy-nine strains grew in a minimal medium with ammonium sulphate and glucose as energy sources, while the rest required additional supplements. In the latter group one strain required methionine and the others yeast nucleic acid. There was no difference in the nutritional requirements of Inaba and Ogawa types and R strains of *V. cholerae*. However, the growth of certain yeast nucleic-acid dependent Inaba strains was considerably improved by the addition of methionine or cystine and in one strain by glutathione.

Examination of certain postulated synthetic units of yeast nucleic acid revealed that the purines hypoxanthine, xanthine, inosine, inosinic acid, adenine, adenosine, adenylic acid, guanine, guanosine and guanylic acid supported the growth of these strains of *Vibrio cholerae*, while the purine precursor 4-amino-5-imidazole-carboxamide and the pyrimidines cytosine and uracil were without effect. Among the former stimulation by hypoxanthine, adenosine and guanosine was marked.

Inactivated sera of certain animals such as rat, mouse, guinea-pig, and horse were unable to support growth of *Vibrio cholerae* in minimal media, while the sera of man, rabbit and goat were effective in this respect. Differences in purine content of these sera might be responsible for this. Optimal purine content in tissues and tissue fluids may be a factor in the pathogenicity of *V. cholerae*.

Certain amino acids were inhibitory to the growth of *Vibrio cholerae* in minimal media supplemented with purines. Norleucine was the most frequent inhibitor followed by norvaline, serine, aminobutyric acid and cystine. The inhibitory patterns were similar to those described in *Escherichia coli*.

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